

„Effects of ocean acidification and paternity on the early life stages of Atlantic cod (*Gadus morhua*) from the south-east Canadian shelf“

Diplomarbeit

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ABSTRACT

Future climate change mainly accelerated by anthropogenic activities which emitted since the industrial era excess amounts of carbon dioxide (CO_2) into the atmosphere, will modify, by CO_2 absorption, ocean water chemistry. It has been shown in many studies that marine organisms are affected by this rise in subsurface seawater carbon dioxide concentration. But today's knowledge about how marine fish species will deal with this possible stressor is not well understood. Their potential acclimatization and adaptive responses are largely unknown. Especially the knowledge that early life stages are more vulnerable than adult fish to adverse environmental conditions forces the need for acidification experiments with a focus on these sensitive stages. A triplicate crossing of gametes from seven males and one female Atlantic cod (*Gadus morhua*) from aquaculture reared fish of south-east Canadian shelf origin produced embryos of seven half sib families, which then were cultured in seawater under three elevated $p\text{CO}_2$ concentrations at 800, 1400, 4000 and 400 μatm as the control, respectively. The $p\text{CO}_2$ incubation started with fertilization and ended after the hatchlings were sampled.

Survival from fertilization to early cleavage (64-128 cell stage) was negatively affected by $p\text{CO}_2$ concentrations, with significant interaction effects with paternity. Elevated $p\text{CO}_2$ concentrations had significant adverse effects on hatching success, but in this case neither paternal or interaction effects were significant. Time to 50 % hatch and body length at time to 50 % hatch could not be evaluated statistically due to high variance in replicates and an insufficient number of hatchlings but based on mean values showed a slight decrease in body length associated with an earlier hatch date in the hypercapnic treatments in relation to the control.

However, ocean acidification is only one stressor associated with climate change and may act synergistically with other environmental factors. Thus, the combination with warming, hypoxia, and perhaps biological shifts in the ecosystem will all contribute as factors to which existing marine fish species and stocks will need to adapt to as existing ocean ecosystems change.

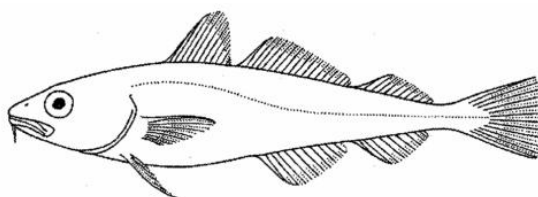


Illustration taken from ICES "Cod" Fishmap.

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1 INTRODUCTION

1.1 Atlantic cod

Cod (*Gadus morhua*) is a member of the Gadidae family in which Atlantic cod (*Gadus morhua morhua*) is a subspecies of the genus *Gadus*, with a wide distribution from the east to the west coasts of the northern Atlantic (see Fig. 3) and can be found at depths down to at least 850 m (Lilly & Carscadden, 2002). Cod have been reported to have attained an age of 25 years and a length of up to 160 cm. Attainment of such impressive sizes is associated with the appropriate diet and environment. Cod larvae start the external food uptake already in the yolk sac stage and feed primarily on nauplii and copepodite stages of copepods beside other smaller zooplankton and phytoplankton species (Last, 1978). After finishing the metamorphosis (also called the O-group stage) they continue to feed on pelagic prey and start slowly to switch their diet from copepods to small fish. With increasing body size Atlantic cod switches its diet preferences from small to larger prey organisms. When cod attain a body length of ~7 cm a move into demersal habitats starts correlated with a diet change to benthic animals, like crustaceans (e.g. *Crangon crangon* and crabs), but the preferred prey remains fish (e.g. gadoids, sandeel, flatfish, and clupeids (Dann, 1989)).

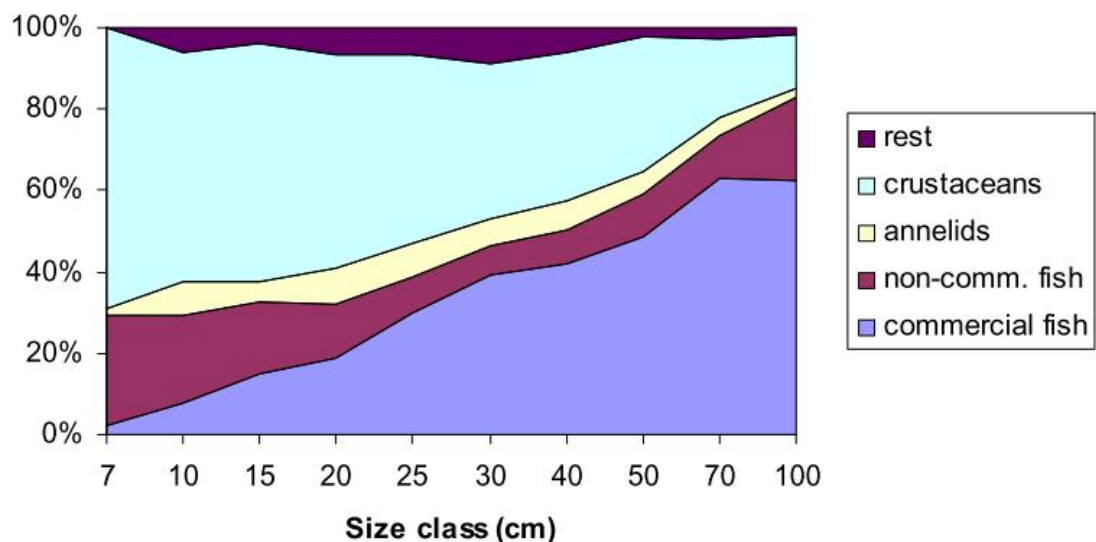


Figure 1: Average cod stomach contents as percentage weight by size class. Data from Dann, 1989. Illustration taken from ICES (2004).

Since centuries, Atlantic cod has been one of the dominant commercial species in Iceland, Spain, Norway and Canada's Newfoundland and has been an ocean resident since ~125 million years. Cod was mainly exploited by land based industry as stock or salt fish and was then sold to the locals or exported all around the world for cash or by the way of exchange, as an important and inexpensive protein source (Kurlansky, 1997). However,

since the early 1990s, Northwest Atlantic cod abundances collapsed to a crucial low level in recruitment since important regions, governed by massive demersal fishing activities and forced by adverse hydrographical conditions and by changes in food web composition and oscillations were no longer or only sparsely populated (Drinkwater, 2005; Frank *et al.*, 2011). International Atlantic cod stocks were depleted by 75 % from 4 million tonnes to lower than 1 million tonnes (ICES, 2004). These collapsing cod stocks led the Canadian Government, in 1992, to adopt fishing regulations for an indefinitely long cod moratorium, 20 years later it seems that the devastated cod stocks, in Canadian waters, remain at very low levels with slight hope in a few locations (Frank *et al.*, 2011) but an uncertainty whether the stocks will ever reach their former levels.

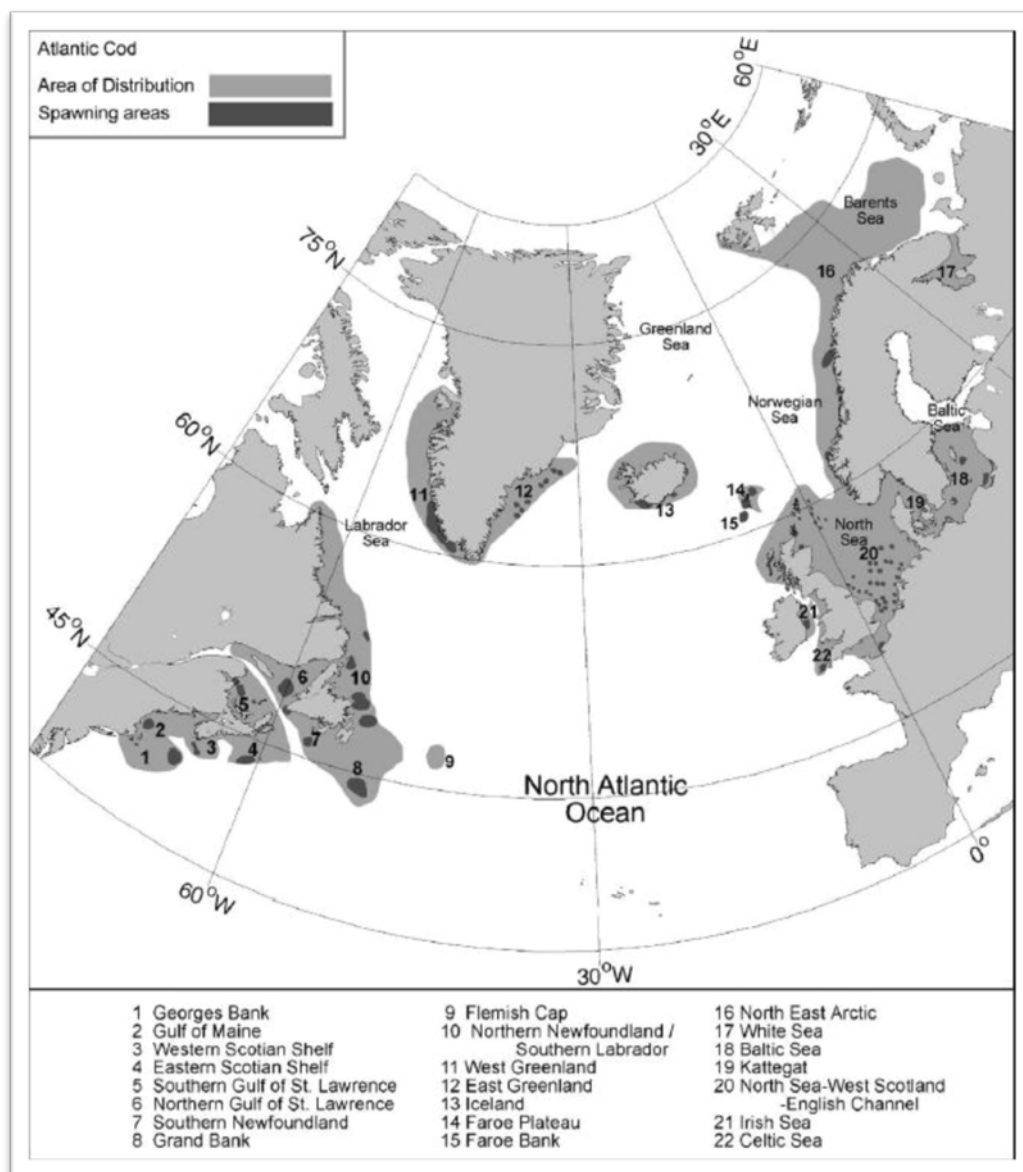


Figure 2: Geographical distribution of Atlantic cod stocks. Taken from Drinkwater (2005).

1.1.1 Spawning behavior

Atlantic cod is a cold water spawner, releasing egg batches within a mean temperature range of 2.5 - 4° C (Lear, 1993), at depth between 20 to 400 meters (Brander, 2005). Spawning activity starts for wild cod, both sexes, at an age between 2 - 8 years and for cod from aquaculture facilities at an age of 2 years (Trippel & Chambers, 1997; Karlsen *et al.*, 2006). Sexual maturity occurs mostly annually, which leads to a release of millions of buoyant pelagic eggs in 15 – 20 batches over a time period for about 50 - 60 days by a single female (Kjesbu, 1989). These numbers vary between different stocks and are closely related to age, size, nutritional status of spawning fish, and/or spawning experience and batch number (Kjesbu, 1989; Kjesbu *et al.*, 1990; Kjørsvik, 1994; Karlsen *et al.*, 2006). Stock differences are also recognized in the timing of spawning seasons which could span the entire year.

1.1.2 Eggs and embryos

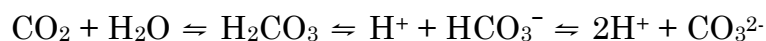
Female Atlantic cod spawn spherical transparent eggs with a mean diameter between 1 - 2 mm and is correlated positively with fish length (Kjesbu, 1989; Brander, 2005). These eggs are spawned from an iso-osmotic environment in the ovary into a hyperosmotic seawater environment (Mangor-Jensen, 1987) where they are floating because of highly hydrated yolk, with a water content up to 90–95% of the egg weight (Bunn *et al.*, 2000; Lubzens *et al.*, 2010) which is protected to diffusion by a water impermeable vitelline membrane (Finn & Kristoffersen, 2007). They are buoyant during the majority of development due to the high water and or low density fluid content in the yolk and the perivitelline space, but just prior to hatch they often slowly start sinking. Cod eggs contain no oil globules in their yolk. The fuel providing embryos with energy for the development are mainly FAA's (Free Amino Acids) (Finn & Kristoffersen, 2007).

Atlantic cod larvae hatch within a size range of 3 to 7.4 mm (Brander, 2005) in which the egg size, amongst other factors, is positively correlated to the age of the female parent (Trippel & Chambers, 1997). Time to hatch is highly variable and depends strongly on water temperature whereas hatching could start between one to eight weeks after fertilization with an average from 21-30 days (Markle & Frost, 1985). Atlantic cod usually spends half a year in the pelagic zones followed by a migration into benthic areas in shallow waters. These areas are often characterized by high hydrological variations like extreme temperature changes between the summer and winter which affect juvenile growth and even mortality rates (Thompson & Riley, 1981; Pepin *et al.*, 1997).

1.2 Ocean acidification

Since the start of industrialization the atmospheric partial pressure of carbon dioxide ($p\text{CO}_2$) has increased by approximately 40 %, and approximately 30 % of this potent greenhouse gas has been diffused into the oceans (Sabine *et al.*, 2004). Whereas some oceanic areas release CO_2 like the Equatorial Pacific (Feely *et al.*, 2006), and vice versa other regions are responsible for the carbon dioxide absorption leading to a total worldwide ocean uptake of 1.3 - 1.8 Pg C year⁻¹. The mid-latitude North Atlantic is the biggest absorber for atmospheric CO_2 (Takahashi *et al.*, 2002) due to the cold water, high primary production, and the continuous export of CO_2 into the deep sphere of the oceans. Models dealing with future climate change predict a two-fold increase in oceanic $p\text{CO}_2$ from ~ 400 to ~ 1000 μatm by the year 2100 (Feely *et al.*, 2009) and another doubling (~ 2000 μatm) is expected to be reached by the year 2300 if future anthropogenic CO_2 emissions are not reduced (Caldeira & Wickett, 2003, 2005). To project these ocean surface $p\text{CO}_2$ changes in pH values the models predict a decrease in 0.2 – 0.3 units in the next hundred years (Orr *et al.*, 2005; Feely *et al.*, 2009; Millero *et al.*, 2009). Nonetheless, the biggest challenge for marine organisms will be the rate as which acidification proceeds. With respect to an extreme ocean acidification period, 55 million years ago (called PETM – The Paleocene-Eocene Thermal Maximum), present day and future acidification is/will occur 10 to 15 times more rapidly (Ridgwell & Schmidt, 2010). Furthermore, the magnitude of ocean acidification in many nutrient rich shallow coastal waters is amplified by the upwelling of CO_2 , produced by the decay of organic matter (Melzner *et al.*, 2012a; Sunda & Cai, 2012). Some of these areas are very important for many marine organisms, such as spawning grounds for wild cod (Drinkwater, 2005), or as shallow settling grounds for shellfish (Feely *et al.*, 2008). These highly acidified waters on the American west coast are already responsible for severe impacts on Pacific oyster (*Crassostrea gigas*) leading to larval die-off's on the US northwest Pacific coast combined with intense structural changes in the local oyster industry (Barton *et al.*, 2012).

To clarify the basic underlying chemical mechanisms causing ocean acidification a short introduction is given: When oceans take up CO_2 , mainly through diffusional exchange with the atmosphere, the carbon dioxide (CO_2) reacts spontaneously, in a equilibration reaction with water, to the very instable carbonic acid (H_2CO_3), followed by two dissociation steps, in which the carbonic acid dissociates into bicarbonate ion (HCO_3^-) and in the second step into carbonate ions (CO_3^{2-}) both reactions are accompanied by proton (H^+) releases.



Reaction direction: “ $\rightarrow \triangleq$ hydration” & “ $\leftarrow \triangleq$ dehydration”

These released protons are then responsible for the pH decrease in seawater. The same underlying chemical mechanisms work in body tissues or body fluids when $p\text{CO}_2$ is increasing, induced by metabolic processes or

due to epidermal diffusion from hypertonic environments like hypercapnic seawater.

1.3 Effects of elevated $p\text{CO}_2$ on fish

When it comes to discussions on biological impacts of ocean acidification the first and foremost focus is on marine calcifying organisms. But to generate a more complex and precise set of reactions on how the variety of marine life will respond to future acidification more marine taxa have to be taken into consideration. Even though an actively regulating taxon like fish is meant to be more robust to future acidification scenarios because of its high metabolic turnover and its powerful ion exchange mechanisms, it still has to invest more energy or at least restructure its internal physiological response structures in order to compensate for the increase (Melzner *et al.*, 2009a; b). Furthermore, to maintain a cutaneous or gill carbon dioxide diffusion gradient during a rise of ambient seawater $p\text{CO}_2$ a fish's internal carbon dioxide partial pressure has to increase (Evans *et al.*, 2005). An increasing number of short-term studies, regarding the effect of acidification on marine fish species, have produced a wide variety of results ranging from very tolerant to strongly affected species, displaying impacts in physiology, histology, morphology and behavior. Just a few long-term studies, dealing with fish and elevated $p\text{CO}_2$, have so far been performed (Melzner *et al.*, 2009b), mostly because of the high experimental effort and cost needed.

Early life stages: Early life stages of the commercially important teleost species lack well-developed systems for acid-base regulation, additionally for oviparous species their yolk energy-storage is finite, until external food uptake begins. Additionally in water spawned gametes like the egg, sperm and resulting zygote the extracellular medium is the seawater whereas this shifts e.g. to blood in later development. This means that during unicellular stages every change of ambient $p\text{CO}_2$ concentration is 100 % reflected in the intracellular lumen. This relative change is weakened when e.g. blood or other extracellular fluids with already high $p\text{CO}_2$ concentrations, regarding to the experienced ones, decrease this relative change (Fig. 3).

All these properties of early life stages make them very sensitive and vulnerable to environmental stress and very dependent on the parental induced configuration. These hypotheses (Fig. 4), regarding to hypercapnic stress, are consistent with a comparison of findings on four marine teleosts which demonstrated that the highest median lethal sensitivity to hypercapnia are the stages from egg to juvenile and not the adult, but with a relative increase in vulnerability with the focus on early embryo cleavage stages prior gastrulation and less on the subsequent embryo stages. While the sensitivity to elevated $p\text{CO}_2$ decreases during the first larval stages, it undergoes an advanced decrease in tolerance from the postflexion larval stages up to the juvenile stage again (Kikkawa *et al.*, 2003; Ishimatsu *et al.*, 2008). The main reasons for these vulnerabilities may be the circumstance that the neutral charged carbon dioxide molecules can easily diffuse from the ambient seawater, via a thin, and regarding to CO_2 diffusion permeable

membrane into the extra- and intracellular lumen. A second pushing factor might be that at these very early cleavage stages there exist no or just a few acid-base regulation mechanisms.

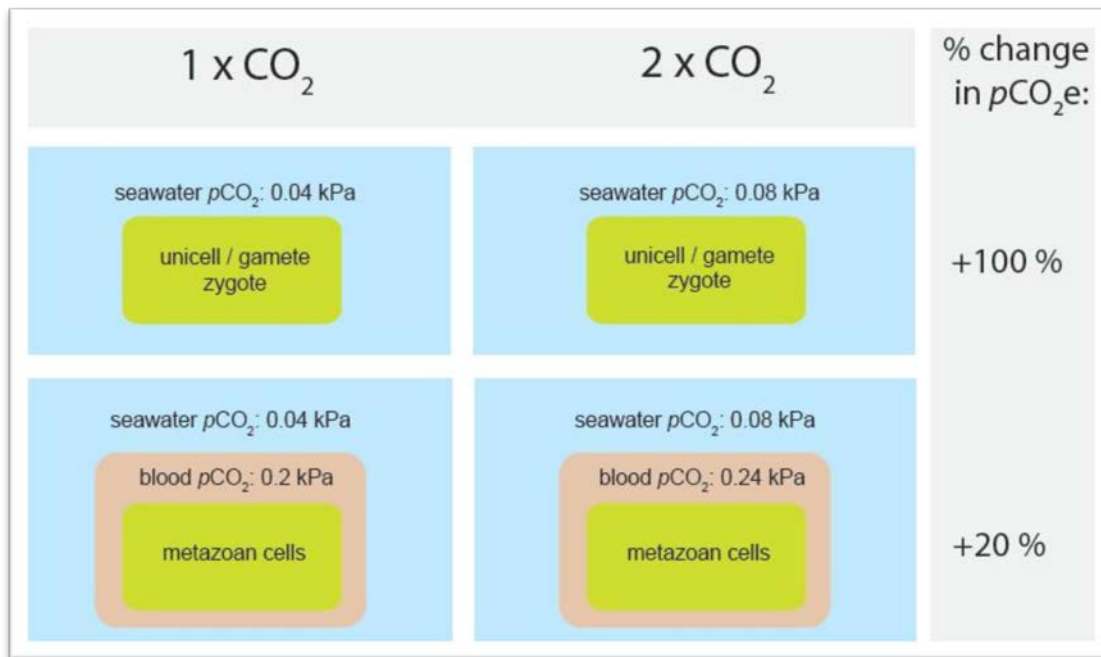


Figure 3: Schematic illustration showing the relative changes in $p\text{CO}_2$ that a cell experiences upon doubling of ocean $p\text{CO}_2$ from 400 to 800 μatm (ca. 0.04 to 0.08 kPa). Unicellular organisms, like fish sperm or a zygote, experience the greatest relative change in $p\text{CO}_2$, as their extracellular environment is the ocean. Metazoan cells are surrounded by extracellular fluid (blood, coelomic fluid or hemolymph), which typically is characterized by $p\text{CO}_2$ values between 1000 and 4000 μatm . An elevation of ocean $p\text{CO}_2$ to 800 μatm , like predicted in future climate change scenarios by the end of this century, would probably only lead to a 20 % increase in $p\text{CO}_2$ of a metazoan with a controlled extracellular $p\text{CO}_2$ of 2000 μatm . The lower relative degree of change in extracellular $p\text{CO}_2$ might render embryo/juvenile/adult metazoans less susceptible to future ocean acidification; however, their gametes might be the most sensitive stages. $p\text{CO}_2\text{e}$: extracellular carbon dioxide partial pressure. Taken from Melzner et al., (2009a).

But step by step, acid-base regulation mechanisms develop coupled to the differentiation of embryonic organs, like the embryo epidermis associated with an accumulation of chloride cells surrounding the yolk sac, that permit increased tolerance to elevated CO_2 concentrations. This increase in tolerance to hypercapnic conditions is maintained until the transition from newly hatched larvae to the juvenile stage when the larvae starts branchial respiration accompanied with a massive increase in diffusion area. In juvenile fish under elevated $p\text{CO}_2$ conditions, the hypercapnic water can flow straight through a large area of gill lamellae, while the only perimeter to CO_2 absorption is the physical diffusion-limit itself (the rate of diffusion through a medium (e.g. cell membranes) is just limited by the rate of production of the transported molecules (e.g. CO_2)). Additionally an expansion of chloride cells in the gills and an increase in cardiac output and blood volume, associated with a rise in red blood cell concentration advances the hydrolysis of CO_2 (Desforges *et al.*, 2002; Perry & Gilmour, 2002). Red blood cells are the major extracellular site for hydration of CO_2 into H^+ and

HCO_3^- and vice versa the dehydration of bicarbonate into CO_2 and water. In studies carried out with Baltic cod (*Gadus morhua callarias*), an exposure to elevated $p\text{CO}_2$ values up to 4000 μatm did not affect motility and speed of sperm, hatching success, survival post hatch, development and otolith size (Schubert, 2009; Frommel *et al.*, 2010, 2012b). However, some major Baltic cod spawning grounds are already hypercapnic, showing $p\text{CO}_2$ concentrations of ~ 1200 μatm and higher (Thomsen *et al.*, 2010; Frommel *et al.*, 2012b). A different picture emerges in the North Atlantic which relative to the Baltic Sea is characterized by relatively stable hydrological parameters. Regarding Atlantic cod, long-term land-based experiments, which investigated the effects on early life stages showed that larval tissues displayed harmful impacts when exposed to CO_2 partial pressures of 1800 and 4000 μatm over a time span of 2 ½ months (Frommel *et al.*, 2012a), but in contrast larval swimming kinematics showed no significant difference in response at low-pH (Maneja *et al.*, 2012). Looking at another teleost species no significant effect of increased $p\text{CO}_2$ on Atlantic herring (*Clupea harengus* L.) fertilization was detected but significant effects were noted on the protein biosynthesis capacity (decreasing RNA/DNA ratio with increasing $p\text{CO}_2$) and significant increases in the otolith lapillus area (ear stones) (Franke & Clemmesen, 2011). In the estuarine fish species *Menidia beryllina*, $p\text{CO}_2$ levels of ~ 1000 μatm induced increased egg and larval mortality while the surviving larvae were affected by reduced growth rates (Baumann *et al.*, 2011). Juveniles of the tropical coral reef fish *Acanthochromis polyacanthus* showed a tolerance to elevated $p\text{CO}_2$ (850 μatm) in growth, survival, skeletal development and ear stone calcification (Munday *et al.*, 2011) though it is important to note that in this study gamete and embryonic stages were not investigated. Another Australian study showed adverse behavioral changes in juvenile coral reef fish, such as reduced food consumption and foraging, when exposed to the combination of elevated temperature and elevated $p\text{CO}_2$ (Nowicki *et al.*, 2012). Elevated $p\text{CO}_2$ values (~ 900 μatm) were shown to affect behavior, this was presented in a study on two coral reef fish species where the olfactory senses were disturbed leading to a behavior that could make it easier for predators to hunt the incautious prey (Nilsson *et al.*, 2012).

Adult stages: A decrease in sensitivity to hypercapnic conditions in adult stages could be explained by their well-developed ion exchange and acid-base regulatory mechanisms, their possibility to replenish their energy storage by external food consumption which is essential during time spans with high physiological stress and the fact of an increase in extracellular fluid quantity which goes along with a higher buffer capacity for CO_2 (Melzner *et al.*, 2009a). This increasing tolerance to CO_2 -enriched water is also shown in research findings on marine teleosts comparing the sensitivity of early ontogeny stages vs. adult stages to sublethal and lethal concentrations of $p\text{CO}_2$ (Ishimatsu *et al.*, 2004, 2008)

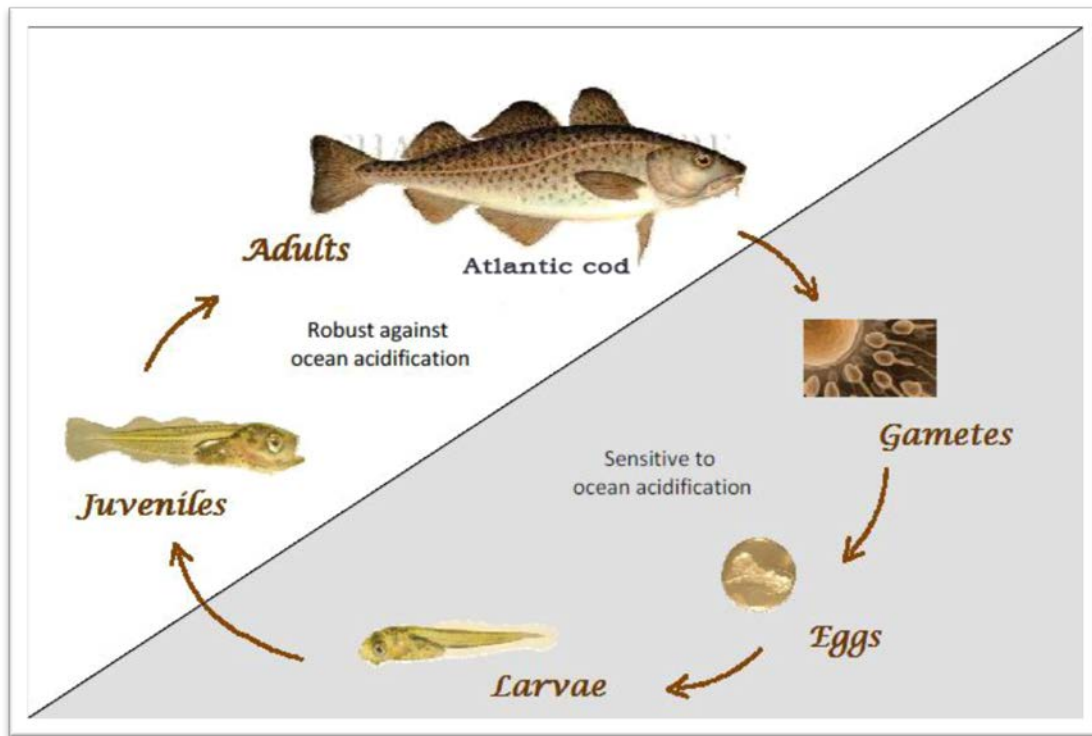


Figure 4: Hypothesis on the sensitivity of ocean acidification at different life stages of cod. Taken from Frommel, (2012a).

1.4 Acid-base regulation in marine fish

Ocean acidification can constrain marine life to expend more fuel on regaining and sustaining their inner pH homeostasis, causing a reduction in expended energy to other bodily processes like somatic development and offspring production.

In animals that extract oxygen from water, aqueous carbon dioxide has the ability to diffuse very fast and easily into extracellular fluids through the branchial epithelium and vice versa CO_2 could diffuse easily out of the organism when plasma $p\text{CO}_2$ increases due to metabolic production, in normocapnic conditions. However, once CO_2 enters into the fluids or tissues, it immediately dissociates into protons and bicarbonate ions, accompanied by a decrease in cytosol and blood pH (Evans *et al.*, 2005). Most fish species possess several compensatory ways to regulate their extracellular acid-base concentrations during respiratory and or metabolic hypercapnia. Aquatic animals have a well-adapted system to accomplish the net transport of molecules which are involved in acid-base regulation among the organism and the environment (Pörtner *et al.*, 2004). The first immediate physiological response to acidosis is divided into two different buffer systems, the CO_2 -bicarbonate and the non-bicarbonate system. Because the CO_2 -bicarbonate system is limited in marine water-breathers due to a weak $p\text{CO}_2$ diffusion gradient between the tissues and the seawater (Heisler, 1986), the non-bicarbonate system becomes the big player in the first reaction to an acidotic pH shift. In this system, the protonation of amino

acid side chains is the main underlying buffer mechanism (Melzner *et al.*, 2009a).

For longer incubation time spans, like for hours or days, a modification in the net exchange of acid-base significant molecules between the animal and the environment is maintained (Evans *et al.*, 2005; Melzner *et al.*, 2009a). On the other hand, a relative high $p\text{CO}_2$ concentration already exists in the extracellular fluids of some teleost species with values between 3000 to 4900 μatm (Melzner *et al.*, 2009a). Fish and other active species have a stockpile of buffers, like bicarbonate, to re-establish their plasma pH (~ 7.8), such as for example a response to short-time hypercapnic conditions or for a possible temporary metabolic pH drop, resulting from prolonged powerful swimming, these same mechanisms are observed in high athletic exposures, due to anaerobic muscle fibre contraction, fueled by ATP (Evans *et al.*, 2005; Melzner *et al.*, 2009a). These processes produce an extra load of protons accumulating in the muscle tissues and demanding further buffer capacities. Some species can store a sufficient amount of buffers to compensate for an unbalanced internal pH system for days or weeks (Melzner *et al.*, 2009a). One example is the spotted wolffish (*Anarhichas minor*) which has a relatively large reservoir of buffers, located in their tissues (Foss *et al.*, 2003). However, neither of these concepts is designed to handle a chronic dose of low pH since a sustained use of these mechanisms sacrifices essential energy. Therefore, it is expected that a relative fast and prolonged change in seawater $p\text{CO}_2$ concentration can affect basic life functions, for instance the assembling of proteins, the preservation of a powerful immune system or even behavioral changes (Pörtner *et al.*, 2004; Pörtner & Peck, 2010). In freshwater and saltwater teleosts, the ionic adjustment mainly takes place in highly specialized ionocytes called mitochondrial-rich cells (MRC - for teleosts were previously also called chloride cells). They are located primarily in the branchial epithelium - the site in which more than 90 % of the acid-base ion exchange takes place, beside their appearance in the kidney, intestine and in some seawater teleost species in special skin areas like the gill cover (Pörtner *et al.*, 2004; Evans *et al.*, 2005). This acid-base regulatory system is characterized by two major mechanisms, the enzymatic platform and the ion-transport protein stage. The crucial enzymatic player is the ubiquitous carbonic anhydrase (CA) and its many isoforms, catalyzing de- and hydration reactions in ionic regulation processes for metabolic and or respiratory compensation. CA is responsible for the hydration of CO_2 into H^+ and HCO_3^- and vice versa the dehydration of bicarbonate into CO_2 and water (Geers & Gros, 2000; Esbaugh *et al.*, 2004). In the case of a high cytosolic CO_2 concentration, may be due to hypercapnia, the products of hydration, HCO_3^- and H^+ , could be exported, in exchange with Cl^- , to the water or the lumen, respectively. Marine teleosts just contain cytosolic CA (CAc) in MRC and in red blood cells but no CA is located in the extracellular plasma like in some elasmobranch species (Henry & Swenson, 2000; Melzner *et al.*, 2009a). Responsible for the cellular import and export of unwanted or wanted ions are the active and passive ion-transport proteins and ion-channel proteins. Hypotheses for marine fish assume that the emerging products of hydration,

e.g. the protons, exit through apical located electroneutral Na^+/H^+ exchangers (NHE) to the ambient seawater in substitution for Na^+ uptake which is then actively pumped against the osmotic gradient across the cytosol, maintained by the basolateral located Na^+/K^+ -ATPase (NKA), into the extracellular fluids. The resulting high extracellular Na^+ concentration generates a positive transepithelial potential which then might extrude the ions through porous tight-junctions from extracellular fluids into the aquatic environment (Deigweier *et al.*, 2008)

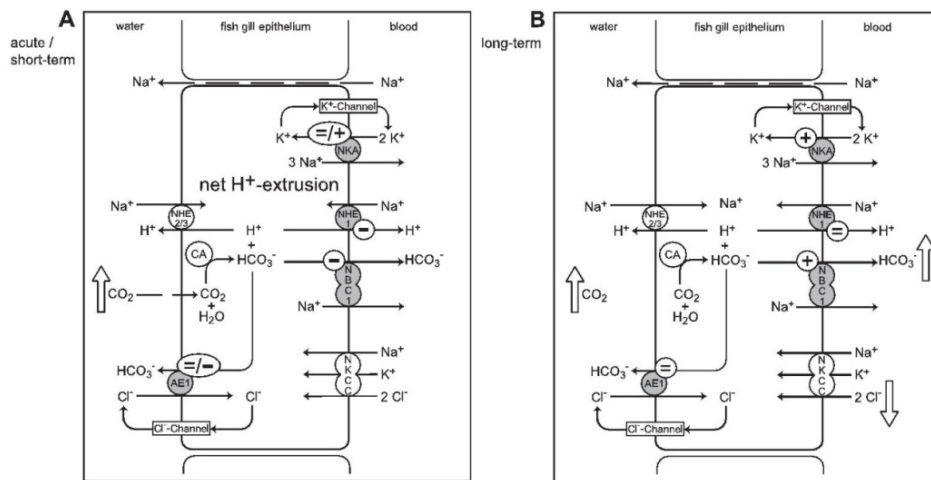


Figure 5: Preliminary model of acute/short-term (A) and long-term (B) adjustments to environmental hypercapnia in the gills of seawater teleost [modified after (Claiborne *et al.*, 2002) and (Evans *et al.*, 2005)]. CO_2 is hydrated by carbonic anhydrase (CA) after diffusive entry resulting in HCO_3^- and H^+ . (A): Acute pH compensation is achieved by non-bicarbonate buffering together with net H^+ extrusion supported by transitional down-regulation (-) of the basolateral Na^+/H^+ exchanger (NHE1) and $\text{Na}^+/\text{HCO}_3^-$ cotransporter (NBC1). Na^+/K^+ -ATPase (NKA) expression is maintained and then upregulated (=/+). A delayed down-regulation (=/-) of apical $\text{Cl}^-/\text{HCO}_3^-$ anion exchanger (AE1) supports the maintenance of higher bicarbonate levels in the cell and plasma when extracellular pH is already restored. During long-term compensation (B), net accumulation of extracellular HCO_3^- is supported by an increase (+) in the abundance of basolateral NBC1 and the maintenance (=) of AE1 and NHE1 levels compared to controls. Net Cl^- decrease in blood may be mediated by a basolateral $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC) and apical Cl^- channels. NHE1 is operating at control levels under long-term steady state conditions. Net proton extrusion is possibly achieved by apical NHE2/3. The driving force for the new steady state is provided by elevated Na^+/K^+ -ATPase levels in the basolateral membrane. Excess Na^+ can diffuse via leaky tight junctions into the surrounding water. Taken from Deigweier *et al.*, (2008).

Early life stages in teleost fish have no differentiated gill apparatus but do already have differentiated cells which are cutaneous ionocytes located mainly in areas around the yolk sac responsible for acid-base regulation and ionic content (Hwang, 1989; Hiroi *et al.*, 1999; Lin & Hwang, 2004; Lin *et al.*, 2006; Bodenstein, 2012). Earlier stages starting e.g. from 1k-cell stages till the end of epiboly lack well developed acid-base regulation cells, whereas intracellular acid-base regulation relies on a relatively high intracellular bicarbonate or non-bicarbonate buffer capacity (intercellular pH of ~ 8 (Heisler, 1986)) and on active ion transport proteins located on the outer cell layer but state-of-the-art knowledge is very scarce on these mechanisms, additionally very different responses to pH shifts were reported among species (Mölich & Heisler, 2005).

1.5 Parentage

Genetic and non-genetic parental influences on their offspring are manifestations of fitness. In fish populations, progeny heritable traits are under the paternal and maternal control (Donelson *et al.*, 2008, 2009), however egg features are mostly forwarded just from the maternal side (maternal variation) (Burt *et al.*, 2010) (Fig. 6). In the range of ontogeny development, the early life stages from gametes to the start of external food uptake, are the most sensitive time spans and are directly linked to the intrinsic properties mediated by parental inheritance. Offspring predispositions could promote their ability to interact and react to an adverse environment and its changes. Parental effects in teleost fin-fish have been shown on e.g., egg size, egg quality (gonad contamination and condition factor (Kjørsvik *et al.*, 1990)), on fertilization and hatching success (Butts *et al.*, 2009), and on larval length, yolk-sac volume, RNA:DNA ratio, and ear stone (lapillar) area (Bang *et al.*, 2006), hence a sophisticated mate choice could provide an ample range for adaptation and resilience responses (Charmantier & Garant, 2005).

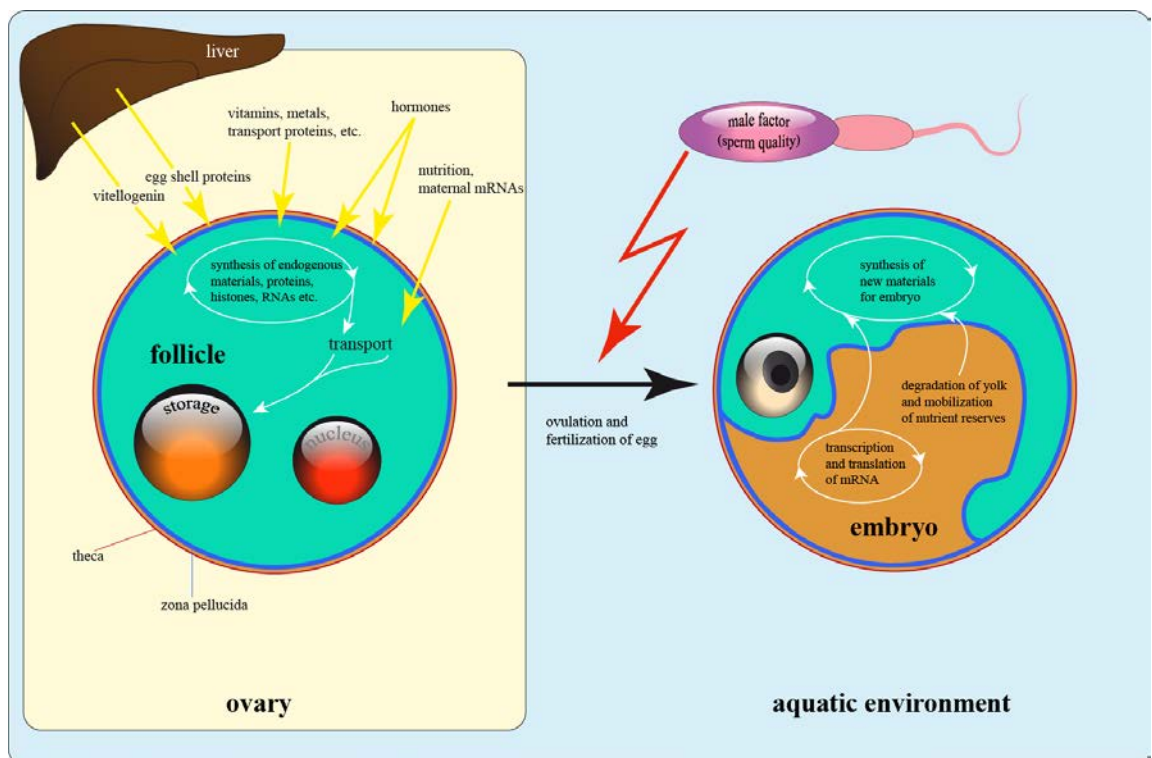


Figure 6: Parental factors and genetic factors that affect egg quality. Factors in yellow are maternal induced and the red flash indicates the paternal input. The underlying information and structure of this illustration was adapted from Brooks *et al.*, (1997).

1.5.1 Maternal implications

Reproductive success in teleost fish is highly correlated with egg quality (Kjørsvik *et al.*, 1990) and it is clear the endogenous determinations of an egg are characterized and forwarded by the mother (see Fig. 6). She governs for e.g. the quality and quantity of yolk ingredients like lipids, vitamins, metals, and protein content in addition to her endocrine influences on egg turnover rates and egg envelope processes. Essential to fertilization and hatching success are also her genetic determinations on the egg and the subsequent embryo like the quality of her transmitted DNA in the nucleus or the content of gene expression related substances in the ooplasm like mRNA's, DNA/RNA polymerases, histones, ribosomes, t-RNA's, and transcription factors (Brooks *et al.*, 1997). Egg and embryonic survival are highly dependent on genetic factors, for example a possible adverse genetic impact could be chromosomal aberrations during early cell cleavages which may lead to irregularly built cell piles (Kjørsvik *et al.*, 1990). But also the non-genetic features, induced by the mother, are very important for egg, embryo and larval development. The egg water content is such a feature that is just defined by the mother, for example low water content leads to a loss of buoyancy and possible descent into an unfavorable hydrographic environment. Poor egg quality could also decrease the resistance to bacteria and fungi when exposed to the environment (Kjørsvik *et al.*, 1990; Brooks *et al.*, 1997; Bunn *et al.*, 2000).

The strength of these maternal effects is partly determined by environmental influences, female age (Solemdal, 1997; Trippel & Chambers, 1997; Berkeley *et al.*, 2004), and also on her genetic predisposition. Maternal variation within a broodstock could influence the mortality rate (Trippel *et al.*, 2005), yolk quality (Kamler, 2005; Bang *et al.*, 2006) and size of larvae at metamorphosis (Green & McCormick, 2005). Whereas important environmental factors affecting these life history traits might be the composition and the amount of the ingested diet and pollutant stress (Kjørsvik *et al.*, 1990; Kjesbu *et al.*, 1996; Brooks *et al.*, 1997; McCormick, 1998). Excessive fishing could create strong selection pressure, for example leading to a contracted adult demography towards small, younger spawners with low egg quality as mid-age fish spawn the best quality of eggs (Kjørsvik *et al.*, 1990; Trippel & Chambers, 1997).

1.5.2 Paternal implications

It is shown that besides the paternal part within the genetic contribution on offspring, sperm qualities, such as sperm motility and sperm density could affect fertilization and thus at least recruitment success (Rakitin *et al.*, 1999a; Trippel, 2003; Gage *et al.*, 2004; Casselman *et al.*, 2006). The importance of paternity was demonstrated on larval size, post hatch growth, yolk size and utilization in a controlled mating experiment including elevated temperature and its interaction effects on half-sib progeny of Atlantic cod (Dahlke, 2011). Paternal effects on embryonic mortality for this

species were also reported in earlier studies (Trippel & Neilson, 1992; Trippel & Morgan, 1994). These kinds of controlled mating experiments revealed paternal impacts on larval growth rate and morphological structures (Rideout *et al.*, 2004). However, the impact dimension in many of these responses is additionally governed by maternal egg quality features and could vary in different development stages (Probst *et al.*, 2006; Dahlke, 2011). An improved fertilization success could be observed when dominant and larger Atlantic cod males were used for reproduction (Hutchings *et al.*, 1999). Spawning behavior is another important factor where the paternal contribution to recruitment success may be for example a mismatch scenario when sperm are released during times where no eggs are spawned (Green, 2008).

In studies which examine parental effects on spawning fish, offspring quality varies greatly among female spawners and this maternal mediated effect may mask paternal effects (Rakitin *et al.*, 1999a). This predominant role of maternal expression in offspring quality and therefore in recruitment success could be the major argument that the number of studies dealing with maternal effects is higher than those dealing with a combination or just with paternal responses (Burt *et al.*, 2010). But a steadily increasing amount of studies that focus on early life stages in commercially important species explore paternal contributions (Trippel, 2003; Rideout *et al.*, 2004; Trippel *et al.*, 2005; Probst *et al.*, 2006; Dahlke, 2011; Houde *et al.*, 2011), showing the urgency in increasing the knowledge on paternal induced effects for the areas of broodstock development in aquaculture and recruitment dynamics in wild stocks. Additionally, such studies with a focus on paternal responses will contribute important answers to predictions on adaptation potential to adverse environmental conditions.

1.6 Objectives of this study

Aim of the study was to investigate how different $p\text{CO}_2$ conditions in combination with paternity affected survival of initial cleavage stages, time to 50 % hatch, hatching success, and size at hatch in Atlantic cod embryos and larvae using a half sib breeding design with one female and seven different males. This setup allows for an evaluation of between family dependent sensitivities to ocean acidification which could be responsible for future adaptation potentials in wild populations.

2 MATERIAL AND METHODS

From February till mid-April 2012 the experiment was carried out in two adjacent sites. The Fisheries and Oceans Biological Station in St. Andrews, New Brunswick, Canada provided the broodstock and the place where gametes were stripped from adult fish. The incubation experiment was set up at the neighbouring Huntsman Marine Science Centre also in St. Andrews.

2.1 Broodstock of the Atlantic cod

The female cod that provided the eggs for the experiment was a F1 progeny of wild caught fish from Browns Bank which is located near the southeastern part of Canada. She was hatched and cultured at the St. Andrews Biological Station (SABS) with an ancestry of the 2009 year-class. The males used to produce the half sib families were also cultured fish from the SABS while their parents were wild caught fish from Browns Bank (2005 year-class). A passive integrated transponder in each fish made it possible to differentiate between related and not related cod. Hence possible inbreeding could be avoided when making crosses. The brood-stock was kept in a 15 m³ flow-through system containing seawater with a salinity of 31 psu and a water temperature range from 3 to 5°C during the spawning season (January to April). Rearing tanks were surrounded by an opaque tarp and equipped with an artificial light source to control light cycles and intensity to be close to the natural ambient ones. The food source for the broodstock was a frozen, freshly thawed diet of northern short-fin squid (*Illex illecebrosus*), Atlantic mackerel (*Scomber scombrus*), Atlantic herring (*Clupea harengus*) and Northern shrimp (*Pandalus borealis*) supplemented with a mix of vitamins.

2.2 Gamete collection

Prior to the stripping procedure, the randomly chosen fish was sedated with MS-222 (Syndel International, Vancouver, BC, Canada) to reduce impacts on the gamete quality from possible high stripping stress (Kjørsvik *et al.*, 1990). To minimize the stress during gamete sampling, it was carried out with high caution and very gentle pressure on the cleaned abdomen. The primary sperm ejaculate and eggs were not used because of possible contamination with seawater, feces and urine or the potential presence of eggs from a previous ovulation process.

Sperm from 7 males was collected in 40 ml dry and clean Pyrex® beakers covered with a lid and stored until fertilization in a 5°C refrigerator. While the stripped egg from several females were poured into separate, clean and dry 3 l plastic beakers, the eggs from the candidate female that appeared to

be of greatest viability (i.e., based on clear yolk, no blood and no signs of overripening (Kjørsvik *et al.*, 1990)) were chosen to be used in the experiment.

2.2.1 Quantification of Sperm density

Spermatocrit of each male was centrifuged for 10 min at 7500 rpm (model Centra® CL3; Thermo IEC, Needham Heights, MA, USA) (Rakitin *et al.*, 1999a) to make comparisons between males and to assure an relative equal sperm density.

Table 1: Summary of male Atlantic cod (*Gadus morhua*) characteristics

Family ID	TL (cm)	Weight (kg)	Collected sperm (ml)	Spermatocrit (%)	Tag ID
1	74,0	9,24	80	33,5	AVID 021 320 334
2	67,2	7,76	50	31	0A00667914
3	71,2	6,59	60	41,5	AVID 021 631 867
4	71,5	7,54	60	31,3	AVID 018 821 560
5	77,5	8,16	30	30	AVID 019 332 606
6	67,0	6,53	25	32,5	0A00714322
7	76,0	7,27	60	36,5	133535262A

2.2.2 Quantification of Egg density

To estimate the abundance of the eggs per ml⁻¹ three random sub-samples with a volume of 0.5 ml each were observed under a microscope (Leica MZ95) immediately after stripping. The number of eggs was enumerated and the average was used for calculations.

Table 2: Summary of female Atlantic cod (*Gadus morhua*) characteristics

	TL (cm)	Weight (kg)	Collected eggs (ml)	Tag ID
Female	58	4,1	1200	AVID 066 020 821

2.3 Fertilization process

Immediately after the viable egg batch had been stripped the gametes were transported in an insulated cooler to a 6°C controlled temperature room at the Huntsman Marine Science Centre. Forty min after stripping all eggs were randomly distributed into 28 600 ml glass beakers (~40 ml eggs per beaker) and each was mixed with 1 ml sperm (dry fertilization method) from different males to create seven half-sibling families under three treatments and one control water condition. After 2 min, 200 – 300 ml seawater was added to activate the fertilization process. For fertilization under acidified conditions the semen was activated with 0.35 µm filtered, UV sterilized and

CO₂ treated (800, 1400 and 4000 μ atm) and untreated seawater for the control, respectively. After a 30 min gamete contact time, the sperm and eggs mixture was poured through a net of a mesh size of 800 μ m and rinsed gently with the associated treatment water. The fertilization procedure was completed by filling the 84 600 ml Pyrex® glass beakers gently with ~500 ml of treatment water and ~15 ml of these eggs, followed by a first incubation time span of 19 hours. Beakers were covered with plastic wrap and rubber band to minimize CO₂ outgassing or absorption. The salinity, temperature and pH of the used seawater was measured shortly before fertilization with a handheld conductivity meter (YSI® 30) and a high precision lab meter (Fisher Scientific accumet® Excel XL60 Meter). All fertilization crossings were completed in less than 3 h post stripping. Insufficient resources were available to conduct separate fertilization success trials with smaller quantities of eggs as described in Butts *et al.* (2010).

2.4 Incubation setup

To help ensure we would have enough fertilized eggs to distribute to the treatments we attempted to fertilize 40 ml of eggs in 600 ml beakers, filled with treated water or control water, respectively. The remaining floating eggs in the beakers on the next day were then further examined. The reason for waiting this time period is that by this time mostly all unfertilized and dead eggs will sink to the bottom due to changes in osmolarity (Davenport *et al.*, 1981). After 19 h incubation, we collected sub-samples from the floating layer, each of about the same egg quantity ($\sim 175 \pm 30$ eggs), to add to each petri dish. Upon inspection, the sub-samples contained not only fertilized eggs but also some unfertilized and dying or already dead fertilized eggs. To deal with this disadvantage we saved an image from each of the 84 loaded petri dishes with a Leica MZ95 Microscope which was equipped with a Q-Imaging MicroPublisher® 3.3 RTV digital camera. The images were saved on a desktop computer by the use of the Q-Capture Pro® 6 software for later analyses to assess the number of each egg type. Immediately after image recording each petri dish was covered with a fly screen mesh (1 mm square mesh) and placed into its assigned incubation tank. This procedure was carried out in a cold room with a temperature of about 6.25°C.

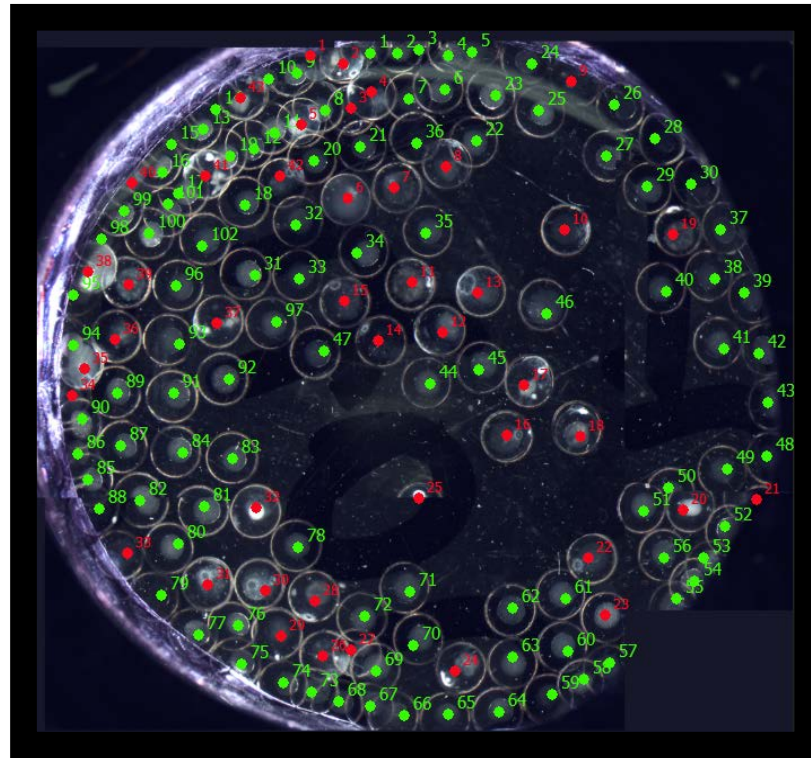


Figure 7: Egg and embryo evaluation via Adobe Photoshop® CS6. Photo shows a petri dish completed with the added eggs/embryos ready for the tanks. Green dots mark fertilized eggs and red dots show unfertilized eggs and eggs/embryos with signs of degradation.

2.4.1 The design of the petri dish system

The circumstance that the atmospheric $p\text{CO}_2$ is close to equal with the $p\text{CO}_2$ concentration in the water, and the fact that Atlantic cod embryos are buoyant and floating at the beaker's water surface especially through the early and mid-phase of development, were the reasons to invent an incubation design which keeps all embryos submerged at a certain water depth. This design assured that the floating embryos especially at the high treatment level were exposed to the designated $p\text{CO}_2$ concentration instead of floating at the surface where the outgassing into the low ambient atmospheric $p\text{CO}_2$ concentration of the cold room presumably lowers the surface water $p\text{CO}_2$ values. To implement this setup we used Pyrex® glass petri dishes with a diameter of 10 cm and a depth of 2 cm. To keep the embryos in the petri dish a soft fly screen mesh (1 mm square mesh) fixed with a rubber band was used as a cover. The mesh size was broad enough to ensure a good water exchange with the surrounding tank water. These petri dishes were then placed into non floating plastic tube racks (Thermo Scientific® Nalgene® Unwire® Test Tube Racks). The alternate position of each petri dish in the racks avoided that no dish covers the underlying one, so as to promote proper water exchange. To hold all the racks in the tanks in the same position they were fastened with cable ties to wood bars mounted at the rim of the tanks. Trial-experiments, performed 2 weeks prior, compared the use of beakers vs. petri dishes (covered by a soft fly screen mesh) as incubation containers, and demonstrated that the embryos were

not harmed by the use of this incubation setup. Hatching success in the petri dish system showed no difference when compared to the beakers.

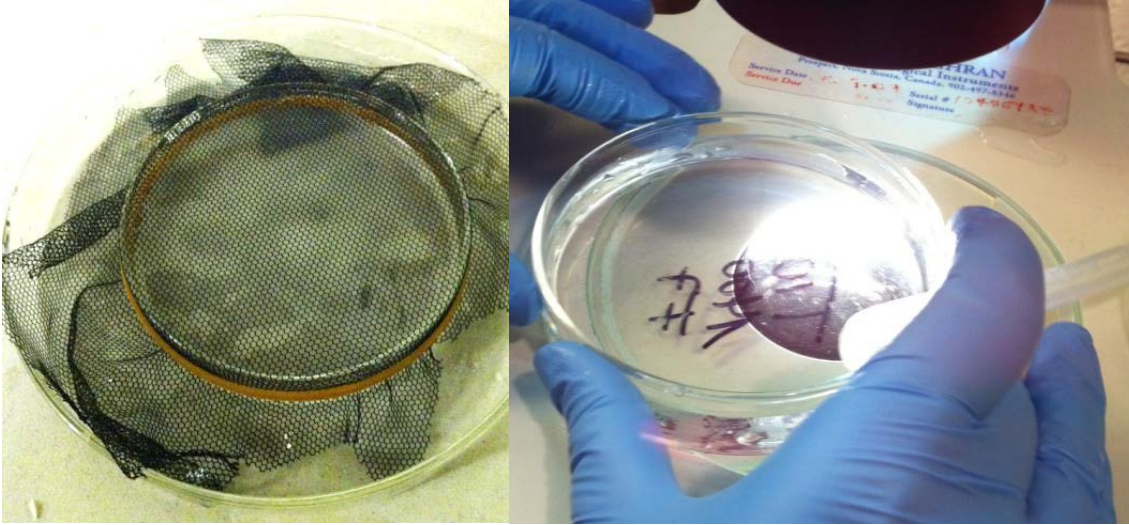


Figure 8: Left photo shows the petri dish covered with a fly screen mesh within a larger sampling petri dish. This was used during the actions of mesh removal and petri dish recovering, respectively, to guarantee that no accident could lead to a loss of eggs. The right photo shows the fast, precise and easy daily counting and removal of dead eggs and embryos under a microscope.

2.4.2 The design of the tank system

The petri dish racks were placed in twelve black round 50 l PVC tanks which, contributing to the different treatments, were randomly distributed in the cold room. In addition, four black 150 l PVC tanks for water change were stored in the cold room containing pre-cooled treatment water or untreated water, respectively. Each exchange water reservoir provided enough water to fill up almost three incubation tanks with fresh water. During the first week of the experiment a water change was conducted every second day. After the first week, this time span was prolonged to three days due to the circumstance that a high amount of embryos and eggs were already removed and the water stayed clean longer, which we could recognize during the sampling procedures.

Each tank was equipped with a small current pump, which kept the water around the petri dishes in motion and provided a more rapid distribution of the injected CO₂. To weaken the outgassing and a possible in gassing of CO₂ into the control treatments all tanks were covered with non-transparent plastic wrap. Shortly before the hatching period these lids were replaced by clear transparent PVC wrap.

All tanks were filled with 0.35 µm filtered and UV treated seawater with a salinity of 31 ±0.5. The daily light cycle in the cold room was set to natural light conditions (~8-10 h light).

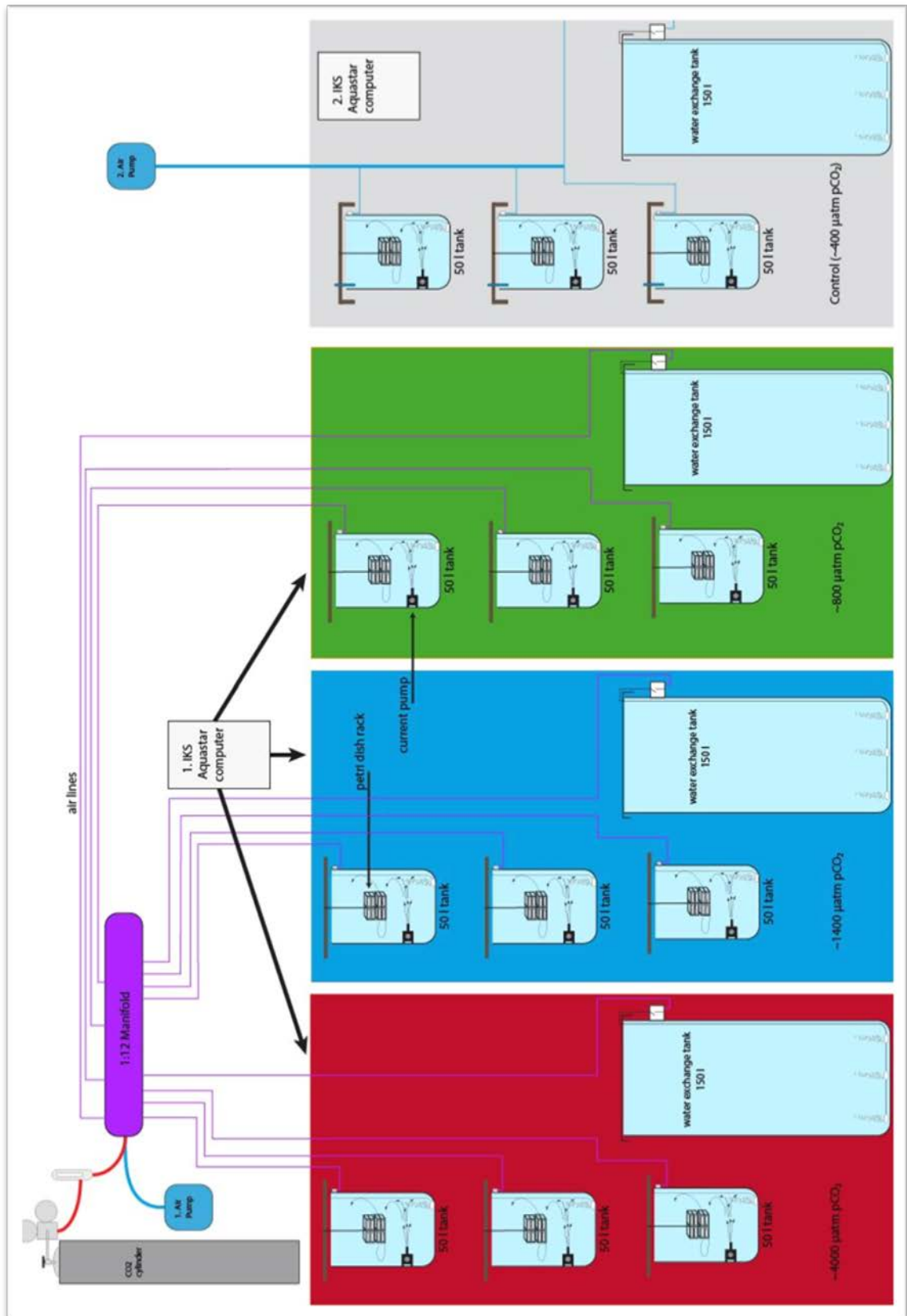


Figure 9: Illustration of the tank setup in the cold room. Two “IKS Aquastar” aquaristic computers controlled the pH of the treatment water. Each tank contained eggs of seven half-sib families, which were distributed separately in seven petri dishes. The locations of the tanks were randomly arranged in the cold room. Air for the CO_2 mixture and for the control aeration was produced by separate air pumps. The air pump for the control tanks was located outside of the building to guarantee an atmospheric $p\text{CO}_2$ without any respiration contamination.

2.4.3 The CO₂ aeration system

An aeration mix of CO₂ and air was produced by the joining of airlines coming from the CO₂ cylinder with airlines coming from an air pump. This setup prevented the introduction of a pure CO₂ injection and additionally provided the tanks with oxygen. The CO₂ cylinder pressure was down regulated by two series-connected pressure regulator valves to create and maintain the target $p\text{CO}_2$ levels in the treatment waters of 800, 1400 and 4000 μatm ($1000 \mu\text{atm} \triangleq 1000 \text{ ppm} \triangleq 0.1 \text{ kPa}$), preventing massive acidification impacts due to an uncontrolled high pressure gas injection. To keep the defined pH-values in the 15 treatment water tanks stable two identical IKS Aquastar[®] computers were used (firmware version 2.26). During the whole incubation time span the Aquastar[®] system continuously monitored the tank water pH-values via high quality IKS glass pH-electrodes and compared the measurements with the programmed pH target ranges. If a pH-electrode sends a signal of a pH-range border crossing, caused by CO₂ outgassing followed by a pH increase, the IKS computer reacts by opening an electromagnetic valve for a certain time span for an injection shot containing the air-CO₂ mixture to recapture the determined pH-value. To keep the CO₂ injection as small as possible the opening time for the valve was set to the shortest available which was 3 sec followed by a waiting time to the next shot of 5 min. To guarantee proper IKS Aquastar pH values, a daily calibration with N.I.S.T. buffers (with an pH of 4 and 7) was conducted and compared additionally with measured pH values delivered by a high precision pH lab meter (Fisher Scientific accumet[®] Excel XL60 Meter) which was calibrated daily with three N.I.S.T. buffers containing a pH of 4, 7 and 10.

For calculations of the carbonate chemistry values for the different $p\text{CO}_2$ treatments, untreated but filtered (0.35 μm) and UV sterilized water samples were analyzed to estimate the C_T (total dissolved inorganic carbon \triangleq DIC) and the A_T (total alkalinity \triangleq TA). The carbonate chemistry lab work was conducted by Darlene Childs at Bedford Institute of Oceanography in Dartmouth, Nova Scotia, Canada. A_T was determined by a titration method with hydrochloric acid (HCl) using a least-squares procedure based on a modified Gran approach (Haraldsson *et al.*, 1997). The titration system consisted of a Metrohm Brinkmann 655 Dosimat[®] titrator and a pH meter (Orion 520A). The seawater samples and the volumetric delivery pipette were thermostated to 25 ± 1 °C using a circulating temperature bath. The electrode, Ross pH electrode (ORION 8103BNUWP) was used to measure the emf during the sample titration. Seawater samples were titrated with diluted HCl made up in a sodium chloride solution in order to yield a total ionic strength similar to that of the seawater sample (Haraldsson *et al.*, 1997). C_T was determined by a SOMMA autoanalyzer, a gas extraction coupled with a coulometric method with photometric detection (Johnson *et al.*, 1993). Precision for A_T and C_T measurements were calculated from replicate samples of certified reference materials (CRM) measured numerous (3) times over the duration of the analysis and ranged from 0.1 % to 1.0 %. Accuracy was within 1 % and results were corrected externally

using a CRM provided by Andrew Dickson at Scripps Institute of Oceanography.

The means of $[C_T]$, the $[A_T]$, the salinity and the temperature were used to compute the unique pH-values by using the CO2SYS program (Lewis & Wallace, 1998). Dissociation constants K_1 and K_2 (Mehrbach *et al.*, 1973; Dickson & Millero, 1987), $KHSO_4$ dissociation constant (Dickson, 1990) and the seawater scale $[mol\ kg^{-1}\ SW]$ were used.

2.5 Counting and measuring of eggs and embryos

Note the terminology used is such that eggs refer to those expelled from the female and were distributed to the beakers for fertilization. A fertilized egg is referred to as an embryo and this term is used until the embryo hatches and then is referred to as a larva or hatchling. Within the first day or so, one is able to detect unfertilized eggs as well as viable embryos and dying or dead embryos which show previous signs of cell cleavage and viability but at the time of observation are non-viable. As time passes, the occurrence of an unfertilized egg would be very remote as these typically quickly sink to the beaker bottom. So, afterwards any dead would presumably be dead embryos. For egg and embryo quantity and quality estimations and for photographic documentation a Leica MZ95 microscope, a Q-Imaging MicroPublisher® 3.3 RTV digital camera, and for image storing the Q-Capture Pro® 6 software, were used. To estimate the exact number of fertilized eggs, 19 hours post fertilization, an image of each petri dish was recorded and the eggs/embryos were counted by the use of the software Adobe Photoshop® CS6. A viable fertilized egg was defined by a clear transparency, cell cleavage and showed no milky discolorations. On the other hand, dying or dead eggs and embryos were identified by a broken egg chorion or signs of milky spots and malformations on the egg membrane or on the embryo, respectively (defined as nonviable). An unfertilized egg showed a clean distributed opacity with no evidence of cell cleavage which were also included into the definition of being nonviable (Fig. 10). The fact that just a subsample of floating eggs and embryos in each beaker was enumerated and the remaining eggs and embryos in the beaker were not counted could lead to an underestimation of fertilization success. The reason for taking just sub-samples was to avoid overfilling the petri dishes and to keep the numbers of eggs/embryos per petri dish constant. A high density of eggs and embryos within a petri dish could be harmful e.g. bacterial infections could rise and spread more easily. The reason for not counting the remaining eggs and embryos in the beakers after the removal of the subsample was due to a lack of manpower. Additionally, regarding to fertilization success estimations, which regularly take place in 4-16 cell stage, it has to be mentioned that after 19 h the embryos were already between the 64-128 cell stages (Fig. 10) and had already experienced 19 h of elevated pCO_2 concentrations and this exposure could have impacted the embryos. However, this affected all petri dishes but to deal with this threat of underestimation in fertilization success we had to redefine fertilization success to viable embryos 19 h post fertilization (hpf)

as a relative measure of fertilized eggs in combination with the possible impacts on mortality during the period leading up to 64 to 128 cells stages. These counts of viable embryos (no signs of degradation after 19 h incubation) were also used as the start number for the estimation of which hatching success is based on. Results from differentiation within nonviable embryos (unfertilized vs. fertilized but dying or dead) were excluded from our main assessments due to insecurities in estimation, because in some cases of high egg degradation we were unable to differentiate between dead unfertilized egg and a dead embryo. Nonviable embryos and/or hatched larvae were collected daily by syringe (Fig. 8, left image).

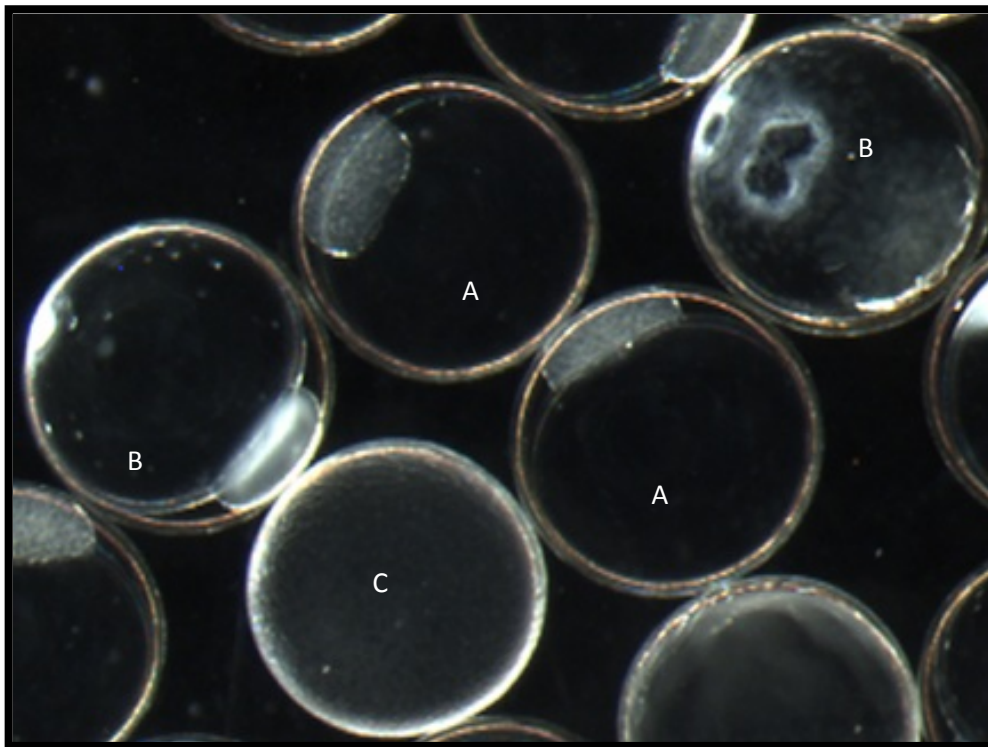


Figure 10: Description of how eggs were quantified, 19 hpf and incubation in beakers, used as the starting count for later estimations like viable embryos 19 hpf or hatching success. (A): Eggs are fertilized, already in a 64 to 128 cell cleavage stage and show no visible signs of degradation and therefore were enumerated for later experimental evaluation, (B): eggs are fertilized or unfertilized but already showing visible marks of degradation like white craters and dots on the egg membranes or on the embryos. (C): Unfertilized egg, characterized by a clean slight opacity. (B) + (C) were not included in the start count for viable embryos 19 hpf and hatching success.

Every petri dish was checked daily through the experimental period. The egg/embryo counting and sampling, as well as the hatched larval counting and sampling were divided into three different procedures governed by the sensitivities and requirements of the embryos and larvae. It was differentiated between incubation periods with very sensitive embryo stages and incubation periods with more robust embryo stages. For a very sensitive sampling each petri dish, one by one, was taken out of a randomly chosen tank while the remaining petri dishes of the belonging petri dish rack were always put back into the tank. The chosen petri dish was carefully placed under a microscope and the dead embryos were counted and

removed. Afterwards, the petri dish was placed back into the tank without a dish change or a cleaning procedure. With this kind of sampling only half of the tank water (~25 l) was changed with fresh water from the appropriate exchange water tank. The sampling procedures during the robust embryo stages differ as follows: the petri dish rack was completely taken out of the tank and placed on a table followed by an investigation of all seven petri dishes. They were placed one by one under the microscope for sampling and counting. After finishing the sampling of each petri dish, the embryos and the water were carefully poured into a fresh and clean petri dish. During the sampling procedure, the regarding tank was cleaned and filled with fresh water from the exchange water tank containing the same $p\text{CO}_2$ concentration as discarded. The third kind of sampling started after the first hatchlings appeared. Immediately, all embryo containing petri dishes were poured carefully into 600 ml beakers filled with fresh treatment water, the larvae were enumerated and removed for measurements (see below) This change in the system was to avoid that larvae could be overseen when sticking on the petri dish mesh-cover and secondly to avoid stressing the larvae by the mesh. Additionally no restriction on the larva's possibility to swim to the surface was wanted. From there on the beakers were sampled once a day to search for hatched larvae, accompanied by water exchange. After collecting the hatched larvae a random subsample of up to a maximum of five were photographed by using a Leica MZ95 microscope with the mounted camera. Between sampling dates, beakers were covered with a clear plastic foil to avoid in or outgassing of CO_2 . The hatched living larvae were photographed directly after removal and measured via the image analysis program ImageJ®, version 1.46r. Standard larval length (SL) was measured from the urostyle to the tip of the upper jaw (Fig. 11).

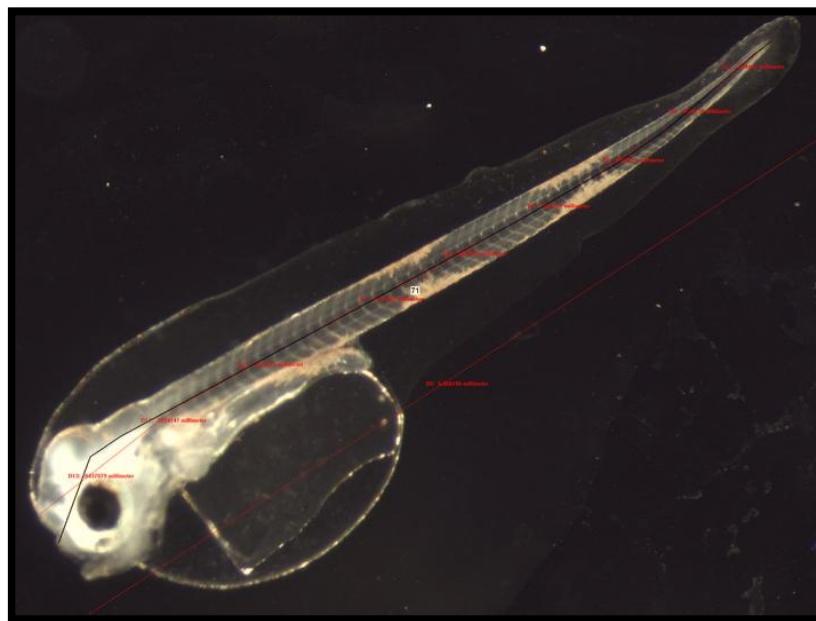


Figure 11: Illustration of a standard length (SL) measurement of a newly hatched larva via the photo analyzing software ImageJ® (black line denotes the measuring track). The red line was taken direct during image capturing and was used as a reference distance.

“Time to 50 % hatch” was specified as the time span in days after fertilization until the point when 50 % of all larvae hatched. The values for these points were obtained by eye from the cumulative hatch plots. The pH, the temperature and the salinity of the treatment water was measured and recorded twice a day, using a daily calibrated lab pH meter (Fisher Scientific accumet® Excel XL60 Meter) and a handheld conductivity meter (YSI® 30). First measurements were taken before the daily IKS Aquastar® pH probe calibration and again after the embryo or larvae sampling procedures. Supplementary the IKS Aquastar® system logged the measured tank pH values continuously, via network, on a desktop computer using the IKS aquaSoft® software, version 2.26.21.

2.6 Statistics

For data based on viable embryos 19 hpf and hatching success tests for homogeneity of variance and normality using residual analysis were carried out with the software R (version 2.15.2, Copyright 2011, The R Foundation for Statistical Computing). If the data met the ANOVA assumptions a multifactorial ANOVA and Tukey HSD post hoc tests were performed with the software Statistica (version 10, Copyright StatSoft Inc., 2011) to test for significant effects of $p\text{CO}_2$ and paternity as well as the potential interaction effect of both parameters on viable embryos 19 hpf and hatching success. $p\text{CO}_2$ concentrations were set as fixed factors and paternity as random factors. In order to test for the effect of $p\text{CO}_2$ and paternity and their possible interaction on the cumulative distributions functions of time to 50 % hatch a nonparametric two-sample Kolmogorov-Smirnov test was applied to the replicates of the different treatments. If no difference between the replicates was found a comparison of the medians from the different treatments was carried out using a Mann-Whitney U-test. The Kolmogorov-Smirnov two-sample test compares differences between two distributions, relying on the null hypothesis that the two samples are distributed identically. The test is based on the unsigned maximum differences between the relative cumulative frequency variations of the two samples. Significance level was set to $p < 0.05$ in all tests.

3 RESULTS

The measurements (weight and body length) which were recorded from the used parental fishes are denoted for the seven males in Table 1 and for the female in Table 2. The mean egg diameter (\pm SD), calculated out of 30 eggs, was 1.683 ± 0.028 mm.

3.1 Physical and chemical conditions

Data from daily measurements of temperature, salinity, pH and total dissolved carbon (C_T) (weekly) are shown in Tables 3 to 7. Carbonate chemistry results at the start and the end of the incubation phase were used to calculate pCO_2 and pH, respectively, to compare between the daily pH measurements provided by the IKS Aquastar[®] sensors and a high precision lab meter (Fisher Scientific accumet[®] Excel XL60 Meter). The calculated pH values of the untreated seawater were additionally used to calculate the setup values for the Aquastar[®] computer. The results on total dissolved carbon originate from a single sample analysis, but to guarantee precise results, a three-fold replicated analysis of certified reference materials (CRM), two at the start and one at the end during the series of lab measurements, was performed. For the CO2SYS calculations, the following parameters were used: K1, K2 from Mehrbach *et al.*, 1973 refit by Dickson and Millero, 1987; $KHSO_4$: Dickson; pH Scale: Total scale (mol/kgSW). Due to the circumstance that the results of the A_T determinations led to very confusing results, even between the samples containing the same water, we decided to use C_T and the measured pH for carbonate calculations. Besides sampling or analyzing errors, the differences in A_T concentrations may be based on the use of recirculating water in the pipes of the aquaristic facilities at the Huntsman station. To weaken such polluting impacts and to eliminate possible embryo quality affecting particles the water was primarily filtered by a $1\mu m$ filter and secondarily with a $0.35\mu m$ filter (Flow-Max[®] Pleated Filter Cartridges) followed by a UV-light treatment. Nonetheless, it seems possible that the pipes are a habitat for algae and other organisms which could influence A_C massively, even with the treatment of small scale filtration, however the C_T is not or just slightly affected by such high concentrations of nutrients or of dissolved organic matter contaminations (for further discussion see Riebesell *et al.*, (2010).

Table 3: Results of seawater chemistry, sampled at the end of February 2012, to estimate the basic values for the later IKS Aquastar® setup calculations. The water was pumped from the bottom of a Bay of Fundy estuary, characterized by extreme daily water in- and outflow events due to extreme tidal differences. We analyzed the C_T of the water at the min level of the low tide (LT01, LT02) and the max level of the high tide (HT01, HT02). pCO_2 and A_T are calculated from C_T and pH measurement with CO2SYS.

Sample	Tides	Temperature (°C)	Salinity	pH	C_T ($\mu\text{mol/kgSW}$)	A_T ($\mu\text{mol/kgSW}$)	pCO_2 (μatm)
LT01	Low tide	5,5	30,7	8,02	2036,32	2154,8	404,4
LT02	Low tide	5,5	30,7	8,02	2042,32	2161,1	405,6
HT01	High tide	5,6	30,7	8,00	1979,21	2089,5	412,5
HT02	High tide	5,6	30,7	8,00	2023,28	2135,0	421,7

Table 4: Carbonate chemistry parameters of each treatment tank, sampled at the “start” of the experiment (on 20th of March 2012), pCO_2 and A_T were calculated from C_T and pH measurements using CO2SYS.

Tank #	Treatment target (μatm)	Temperature (°C)	Salinity	pH	C_T ($\mu\text{mol/kgSW}$)	A_T ($\mu\text{mol/kgSW}$)	pCO_2 (μatm)
1	1400	6,2	30,4	7,48	2104,58	2072,82	1469,9
2	400	6,1	30,6	8,03	2016,65	2140,93	392,8
3	800	6,1	30,4	7,64	2071,06	2081,15	1004,9
5	4000	6,1	30,3	7,17	2231,27	2094,45	3086,6
6	400	6,0	30,6	8,03	2011,63	2135,16	391,5
7	400	6,0	30,5	8,03	2010,22	2133,23	391,5
9	800	5,8	30,1	7,66	2068,24	2081,62	957,8
10	1400	5,6	30,1	7,56	2110,01	2096,45	1225,6
11	4000	5,6	30,0	7,04	2293,71	2091,22	4166,9
13	1400	5,7	30,1	7,54	2129,28	2110,53	1295,2
14	800	5,4	30,1	7,61	2082,00	2080,98	1077,6
15	4000	5,3	30,1	7,23	2199,05	2082,53	2653,7

Table 5: Carbonate chemistry parameters of each treatment tank, sampled at the “end” of the experiment (on 3th of April 2012). Additionally a duplicated control water (CW) sample was analyzed, pCO_2 and A_T calculated from C_T and pH measurements with CO2SYS.

Tank #	Treatment target (μatm)	Temperature (°C)	Salinity	pH	C_T ($\mu\text{mol/kgSW}$)	A_T ($\mu\text{mol/kgSW}$)	pCO_2 (μatm)
1	1400	7,5	30,6	7,48	2063,93	2046,03	1360,2
2	400	7,5	30,6	8,03	2026,79	2155,89	407,8
3	800	7,6	30,1	7,64	2010,62	2048,94	803,3
5	4000	6,9	30,3	7,17	2227,36	2061,81	3685,2
6	400	6,5	30,5	8,03	2018,05	2129,43	445,6
7	400	7,0	30,3	8,03	2033,71	2150,54	428,1
9	800	6,9	30,2	7,66	2028,39	2065,46	785,0
10	1400	6,7	30,4	7,56	2082,40	2082,80	1140,8
11	4000	6,7	30,3	7,04	2250,85	2055,87	4227,0
13	1400	6,6	30,7	7,54	2122,45	2098,20	1420,8
14	800	6,5	30,8	7,61	2067,44	2110,02	780,5
15	4000	7,0	30,4	7,23	2232,88	2056,65	3841,2
CW01	400	6,7	30,7	8,00	1967,16	2082,92	412,9
CW02	400	6,7	30,7	8,01	1978,20	2097,82	405,4

Table 6: Carbonate chemistry parameter and pH comparisons. Deviations from the target levels of the different $p\text{CO}_2$ treatments are shown. The comparison of C_T and pH is between the start (Table 4) and the end values (Table 5) of the incubation period.

Tank #	Treatment target (μatm)	$p\text{CO}_{2,\text{end}}$ (μatm)	$\Delta p\text{CO}_2$ (μatm)	$C_{T,\text{end}}$ ($\mu\text{mol/kgSW}$)	ΔC_T ($\mu\text{mol/kgSW}$)	pH_{end}	ΔpH
1	1400	1360,25	-39,75	2063,93	-40,7	7,51	0,03
2	400	407,81	7,81	2026,79	10,1	8,02	-0,01
3	800	803,30	3,30	2010,62	-60,4	7,73	0,09
5	4000	3685,16	-314,84	2227,36	-3,9	7,09	-0,08
6	400	445,59	45,59	2018,05	6,4	7,98	-0,05
7	400	428,13	28,13	2033,71	23,5	8,00	-0,03
9	800	785,01	-14,99	2028,39	-39,9	7,74	0,08
10	1400	1140,83	-259,17	2082,40	-27,6	7,59	0,03
11	4000	4227,01	227,01	2250,85	-42,9	7,03	-0,01
13	1400	1420,84	20,84	2122,45	-6,8	7,50	-0,04
14	800	780,49	-19,51	2067,44	-14,6	7,75	0,14
15	4000	3841,17	-158,83	2232,88	33,8	7,07	-0,16

Table 7: Mean \pm standard deviation of daily measured tank parameters for temperature, salinity and pH. The A_T and the $p\text{CO}_2$ were recalculated by using the mean values from this Table and the T_C concentrations at the termination of the experiment.

Tank #	Treatment target (μatm)	Temperature ($^{\circ}\text{C}$)	Salinity	pH measured	A_T ($\mu\text{mol/kgSW}$)	$p\text{CO}_2$ (μatm)
1	1400	6,86 \pm 0,7	29,9 \pm 0,8	7,54 \pm 0,07	2014,2	1235,8
2	400	6,66 \pm 0,8	30,4 \pm 0,3	7,98 \pm 0,04	2120,1	441,5
3	800	6,76 \pm 0,7	30,2 \pm 0,3	7,71 \pm 0,10	2257,2	932,5
5	4000	6,59 \pm 0,7	29,9 \pm 0,8	7,15 \pm 0,15	1890,2	2914,1
6	400	6,44 \pm 0,8	30,4 \pm 0,2	7,99 \pm 0,03	2144,7	439,9
7	400	6,50 \pm 0,7	30,4 \pm 0,3	7,99 \pm 0,03	2119,3	436,4
9	800	6,47 \pm 0,6	29,9 \pm 0,5	7,73 \pm 0,06	2061,0	811,2
10	1400	6,51 \pm 0,7	30,0 \pm 0,4	7,53 \pm 0,06	2063,3	1318,9
11	4000	6,34 \pm 0,5	30,0 \pm 0,3	7,06 \pm 0,05	2068,0	3915,9
13	1400	6,46 \pm 0,6	29,9 \pm 0,8	7,54 \pm 0,08	2106,5	1296,1
14	800	6,22 \pm 0,6	30,1 \pm 0,5	7,68 \pm 0,08	2088,6	908,7
15	4000	6,12 \pm 0,5	30,0 \pm 0,4	7,10 \pm 0,05	2064,2	3630,0

Oxygen content in the tanks was measured at the beginning, the mid and at the end of the experiment with a handheld optical YSI ProODO® dissolved oxygen meter showing constant dissolved oxygen values from 106 ± 16 % air saturation ($\sim 10.26 \pm 1,6$ mg L⁻¹). During the experimental period the climate room temperature increased by $\sim 1^{\circ}\text{C}$ from $5.5 \pm 0,5$ $^{\circ}\text{C}$ to $6.5 \pm 0,5$ $^{\circ}\text{C}$ affecting all incubation tanks.

3.2 Early viable embryonic development

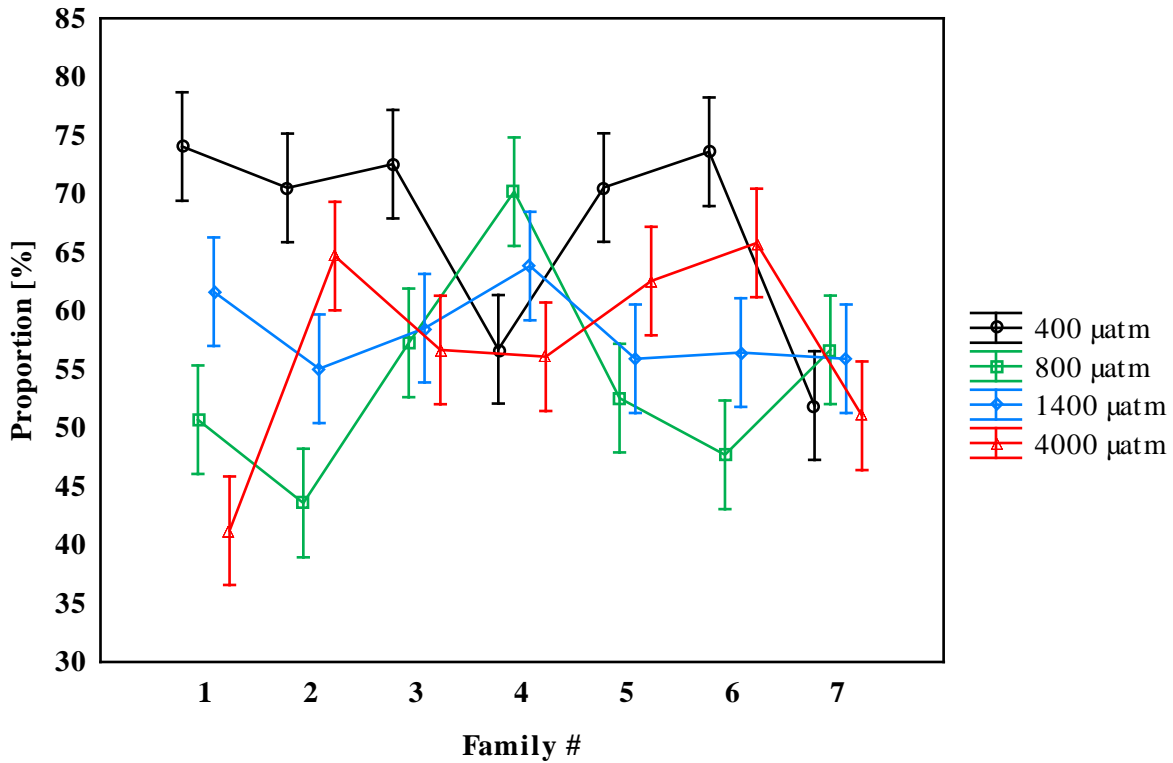


Figure 12: Plot shows the significant ($p < 0.0001$, $F = 12.94$) interaction of $p\text{CO}_2$ and paternity on the mean percentages of viable embryos 19 hours post fertilization (hpf). Error bars denote \pm confidence interval (CI) (95%) with $n = 3$ replicates.

Mean percentages of viable embryos 19 h post fertilization (hpf) were significantly affected by the interaction of $p\text{CO}_2$ with paternity (multifactorial ANOVA: $p < 0.0001$, $F = 12.94$) (Fig. 12). Our results show strong differences between and within families and their responses to elevated $p\text{CO}_2$ such as the proportion of viable embryos from family 1, 2, and 6 differed significantly between treatments, showing mean percentages (\pm SD) of viable embryos in family 1 from 41.2 ± 3.9 % at 4000 μatm to 74.1 ± 2.9 % in the control level. A different pattern emerged in families 3, 4, and 7 where the responses to different treatments were less variable, e.g. family 7 showed just minor differences in mean percentages in all treatment concentrations ranging from 51.0 ± 3.1 to 56.7 ± 2.3 %. Additionally, family-level differences occurred within a treatment. Family 1 showed the strongest impacts at 4000 μatm (41.2 ± 3.8 %) while in families 2 and 6 the proportions were significantly decreased by the 800 μatm treatment (43.6 ± 1.5 % and 47.7 ± 8.6 %), however the same $p\text{CO}_2$ concentration showed in family 4 the highest percentages of viable embryos at about $\sim 70.2 \pm 3.0$ %.

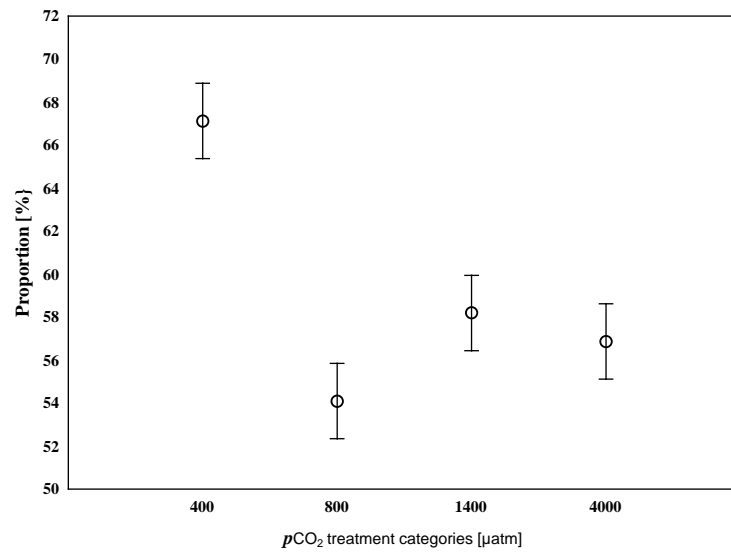


Figure 13: Plot shows the significant ($p = 0.048$, $F = 3.205$) effect of $p\text{CO}_2$ regarding the mean percentage of viable embryos 19 hpf. The 7 half-sib families are combined in each treatment. Error bars denote $\pm\text{CI}$ (95%) with $n = 3$ replicates.

When paternity was excluded significant differences were found between the mean percentage of viable embryos 19 hpf (multifactorial ANOVA: $p = 0.048$, $F = 3.205$) regarding the effects of elevated $p\text{CO}_2$. All three elevated treatments are closer together compared to the control treatment. No significant differences were found between 1400 μatm and the 4000 μatm , but 800, 1400 and 4000 μatm were significantly different from the control. The mean percentages ($\pm\text{SD}$) of viable embryos 19 hpf ranged from $67 \pm 9.3\%$ in the control tanks down to $\sim 54 \pm 8.8\%$ in 800 μatm .

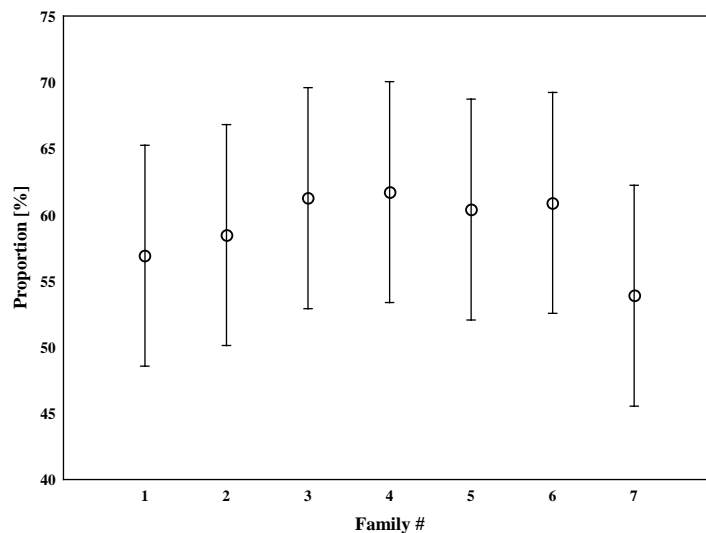


Figure 14: Mean percentage of viable embryos 19 h hpf related to paternal differences between 7 half sibling families without contribution to $p\text{CO}_2$ concentrations showing no significant differences ($p = 0.822$, $F = 0.47$). Error bars denote $\pm\text{CI}$ (95%) with $n = 12$ replicates.

When we focused on paternity, mean percentages of viable embryos 19 hpf showed no significant differences among families (Fig. 14).

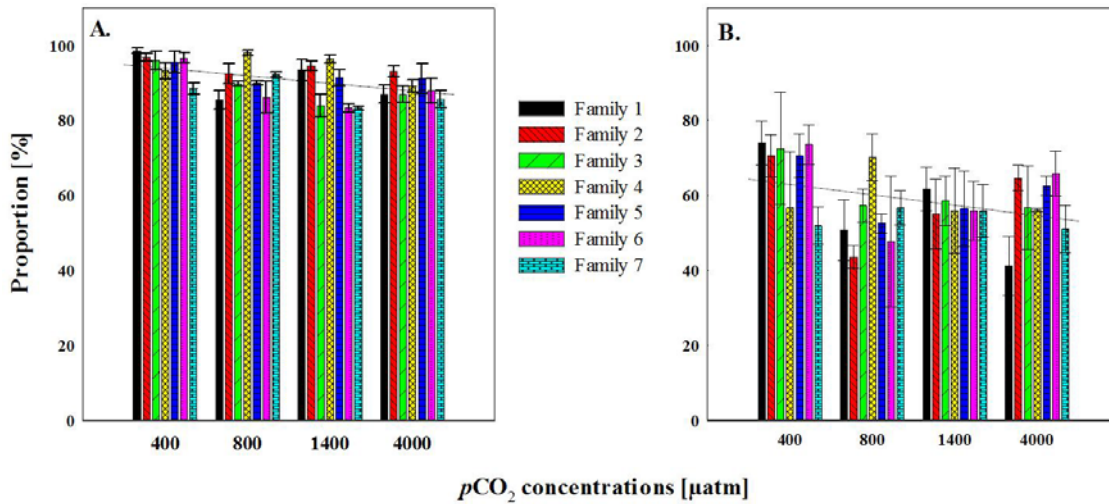


Figure 15: Differentiation between (A) total fertilized eggs (sum of viable embryos and nonviable embryos) and (B) just viable embryos. (A) and (B): Bars report the mean percentage after 19 hpf between families and treatments. Regression line: (A) $r^2 = 0.24$ and (B) $r^2 = 0.13$, error bars denote \pm standard deviation with $n = 3$ replicates.

Even if we decided not to use the total fertilized eggs (sum of viable embryos and nonviable embryos) because of uncertainties e.g. in the visual differentiation between highly degraded unfertilized eggs and highly degraded dead embryos (see chapter 2.5), the results, when adding the nonviable embryos to the viable embryos and define them as total fertilized eggs (Fig. 15 (A)), showed the same or even stronger impacts of elevated $p\text{CO}_2$ concentrations ($r^2 = 0.24$) than we observe for viable embryos 19 hpf ($r^2 = 0.13$, Fig. 15 (B)). We can further confirm these findings if we take a look on the proportion of unfertilized eggs (Fig. 16 (A)) and the proportions of the nonviable embryos (Fig. 16 (B)) where the proportion of unfertilized eggs is significantly ($p = 0.02$, $F = 4.2$) increasing with increasing $p\text{CO}_2$ concentration while no differences in the mean percentages of nonviable eggs between treatments were found (Fig. 17).

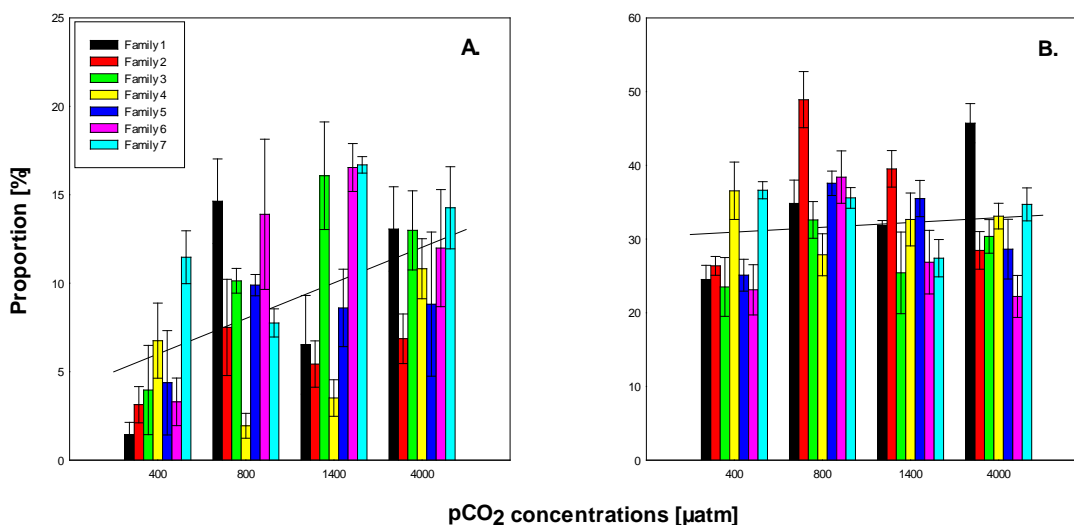


Figure 16: Comparisons between (A) unfertilized eggs and (B) nonviable embryos. Bars show mean percentages 19 hpf between families and treatments. Regression line: (A) $r^2 = 0.24$ and (B) $r^2 = 0.01$, error bars denote \pm standard deviation of $n = 3$ replicates.

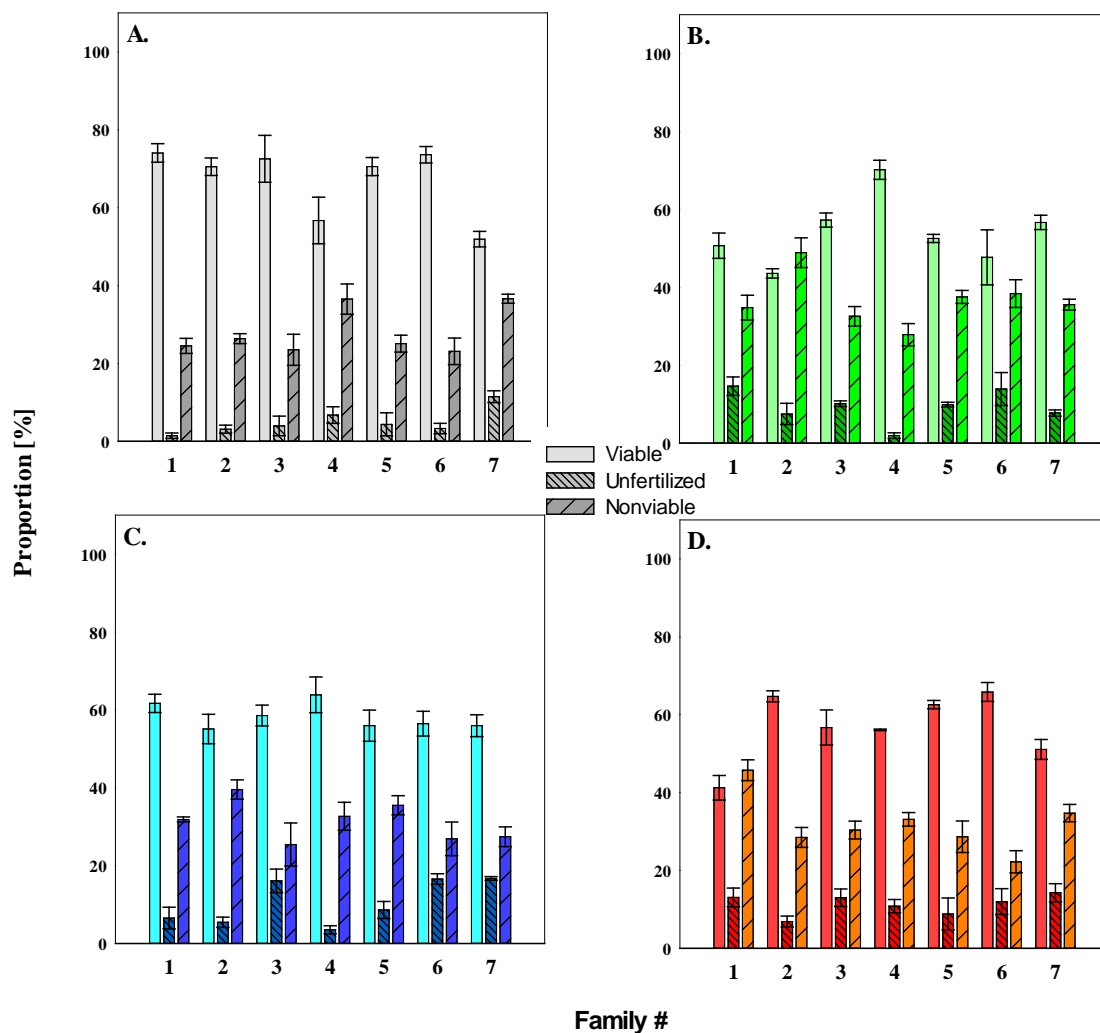


Figure 17: Treatment comparisons of viable embryos, unfertilized eggs and nonviable embryos of each of the seven families. Bars show the mean percentages 19 hpf. Whereas (A) shows the 400, (B) the 800, (C) the 1400, and (D) the 4000 μatm $p\text{CO}_2$ treatment concentration. Error bars denote the \pm standard deviation with $n = 3$ replicates.

3.3 Time to 50 % hatch

Table 8: Summary of nonparametric two-sample Kolmogorov-Smirnov tests which were performed to estimate homogeneity in cumulative differences between family replicates for “time to 50 % hatch”. Green cells with an asterisk (*) denote significant differences between the replicates, red and a (n.s.) denotes no significant differences, and yellow and (n.t.) indicates this case is not tested because of missing values.

Family 1			Family 2			Family 3			Family 4		
400 μ atm	Tank 6	Tank 7	400 μ atm	Tank 6	Tank 7	400 μ atm	Tank 6	Tank 7	400 μ atm	Tank 6	Tank 7
Tank 2	*	*	Tank 2	*	*	Tank 2	*	*	Tank 2	*	*
Tank 6		*	Tank 6		*	Tank 6		*	Tank 6		*
800 μ atm	Tank 9	Tank 14	800 μ atm	Tank 9	Tank 14	800 μ atm	Tank 9	Tank 14	800 μ atm	Tank 9	Tank 14
Tank 3	*	n.s.	Tank 3	n.s.	n.s.	Tank 3	n.s.	n.s.	Tank 3	*	n.s.
Tank 9		*	Tank 9		n.s.	Tank 9		*	Tank 9		n.s.
1400 μ atm	Tank 10	Tank 13	1400 μ atm	Tank 10	Tank 13	1400 μ atm	Tank 10	Tank 13	1400 μ atm	Tank 10	Tank 13
Tank 1	n.s.	n.s.	Tank 1	n.s.	*	Tank 1	n.s.	*	Tank 1	n.s.	n.s.
Tank 10		n.s.	Tank 10		n.s.	Tank 10		*	Tank 10		n.s.
4000 μ atm	Tank 11	Tank 15	4000 μ atm	Tank 11	Tank 15	4000 μ atm	Tank 11	Tank 15	4000 μ atm	Tank 11	Tank 15
Tank 5	n.s.	n.s.	Tank 5	n.s.	n.s.	Tank 5	n.s.	n.t.	Tank 5	n.t.	n.t.
Tank 11		n.s.	Tank 11		n.s.	Tank 11		n.t.	Tank 11		n.s.

Family 5			Family 6			Family 7		
400 μ atm	Tank 6	Tank 7	400 μ atm	Tank 6	Tank 7	400 μ atm	Tank 6	Tank 7
Tank 2	*	*	Tank 2	*	n.t.	Tank 2	*	*
Tank 6		*	Tank 6		n.t.	Tank 6		*
800 μ atm	Tank 9	Tank 14	800 μ atm	Tank 9	Tank 14	800 μ atm	Tank 9	Tank 14
Tank 3	n.t.	n.s.	Tank 3	n.s.	n.s.	Tank 3	n.t.	n.t.
Tank 9		*	Tank 9		n.s.	Tank 9		n.t.
1400 μ atm	Tank 10	Tank 13	1400 μ atm	Tank 10	Tank 13	1400 μ atm	Tank 10	Tank 13
Tank 1	n.t.	n.s.	Tank 1	*	n.s.	Tank 1	*	n.s.
Tank 10		n.t.	Tank 10		n.s.	Tank 10		n.s.
4000 μ atm	Tank 11	Tank 15	4000 μ atm	Tank 11	Tank 15	4000 μ atm	Tank 11	Tank 15
Tank 5	n.s.	n.s.	Tank 5	n.s.	n.s.	Tank 5	n.s.	n.t.
Tank 11		n.s.	Tank 11		n.s.	Tank 11		n.t.

Tests for homogeneity between the replicates of families in time to 50 % hatch showed significant differences, especially between the replicates in the control (400 μ atm) groups (Table 8). For other tanks difficulties in cumulative calculations appeared (see yellow or “n.t.” marks in Table 8) because no hatching occurred in some replicates due to the reason that all embryos in the regarding petri dish had died before hatching. The circumstance that homogeneity in time to 50 % hatch was not given made serious tests for family responses and interaction responses between paternal and treatment effects not possible.

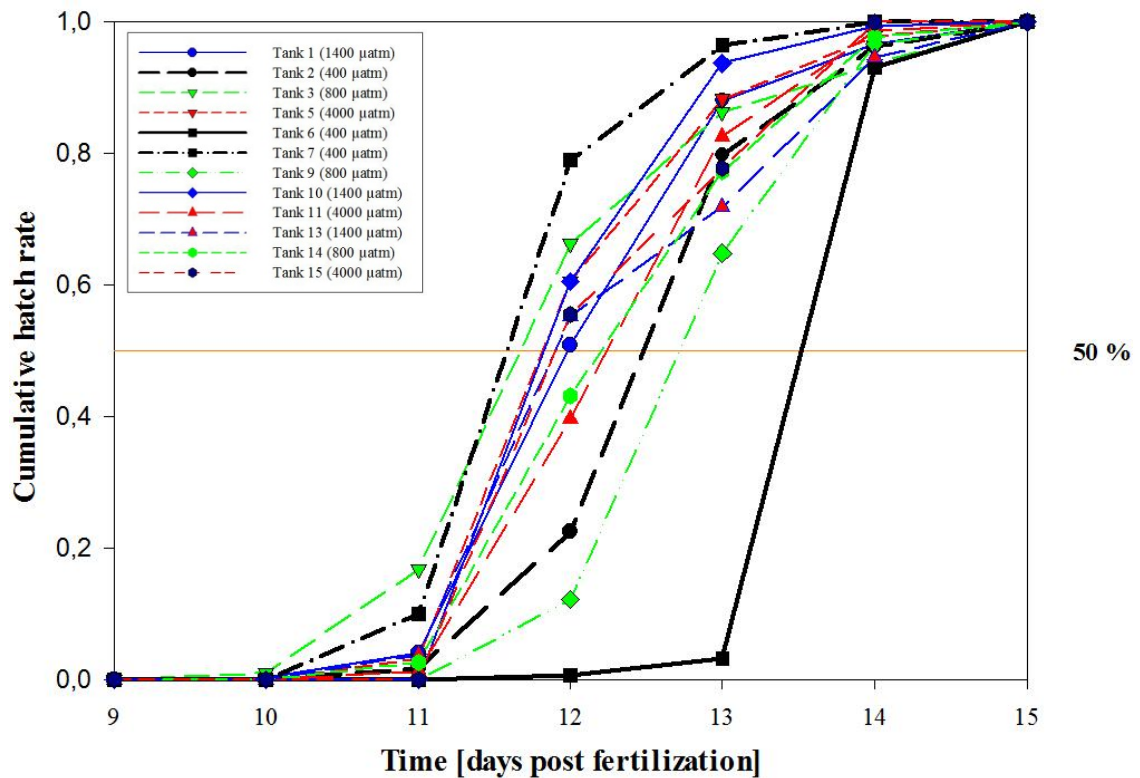


Figure 18: Cumulative hatch in relation to the time of hatch (days post fertilization) showing comparison between the tanks (replicates) and the treatments, paternal effects were not included. The 50 % reference line indicates the percentage when more than half of the larvae within a tank had hatched.

If we used the mean of all seven families per tank and compared the replicates of the treatments, significant differences within the control-tanks appeared (Fig. 18) leading to the same statistical problems like in family replicates comparisons. However, the first larvae hatched at day 10 post fertilization and the date of 50 % hatch ranged between day 11 and 13. The hatching period was finished at day 15 with tank 6 (400 µatm) being the last tank being between day 13 and 14. If we ignore the variance between the treatment replicates because of the small $n = 3$, and calculate the cumulative hatch out of the means from all hatchlings in a treatment (Fig. 19) a slight pattern shows up revealing that the mean 50 % hatch occurred earlier in the elevated $p\text{CO}_2$ treatments (800 µatm at day 12.2, 1400 µatm at day 11.8, 4000 µatm at day 12.0) than in the control (400 µatm at day 12.5) whereas the control showed the highest spread (± 1.97) and 1400 µatm the smallest spread (± 0.11) denoted by \pm confidence intervals in Figure 19.

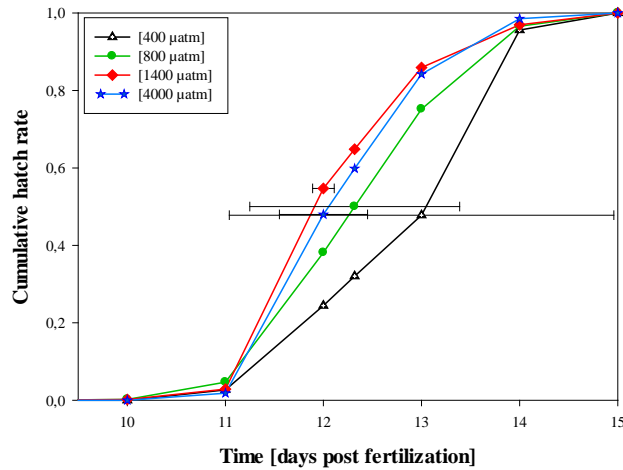


Figure 19: Cumulative hatch in relation to the time of hatch (days post fertilization) showing the differences in time to 50 % hatch between treatments. The data points denote the mean of all hatched eggs per treatment (seven families per tank and each tank 3 times replicated per treatment). The error bars indicate the \pm CI (95%, $n = 3$) for the time when 50 % of the larvae had hatched. The reason that some of the CI's are not precisely plotted on the 50 % reference line (800 and 1400 μatm) is due to better visibility.

Table 9: Summary of days post fertilization (dpf) needed to attain 50% hatch shown for treatment tanks within regard to $p\text{CO}_2$ concentration. All families and replicates combined. Values are taken from the cumulative distribution plot (Fig. 18).

Time to 50% hatch												
Tank #	1	2	3	5	6	7	9	10	11	13	14	15
$p\text{CO}_2$ [μatm]	1400	400	800	4000	400	400	800	1400	4000	1400	800	4000
dpf	11.8	12.5	11.7	11.8	13.5	11.6	12.7	11.8	12.3	11.9	12.2	11.9

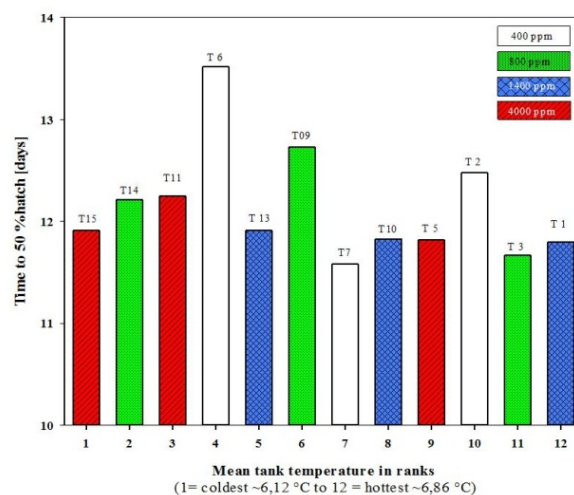


Figure 20: Time to 50 % hatch (dpf) in relation to differences in tank temperature recorded during the incubation time span. Each bar represents the mean percentage of 7 families in one tank. The tank number is noted on top of each bar and the temperature is given in ranks from 1 (coldest: 6.12 ± 0.5) to 12 (warmest: 6.86 ± 0.7).

In relation to the mean differences in tank temperatures that occurred during the entire incubation period no effect of slightly higher elevated temperature on time to 50 % hatch was observed.

3.4 Hatching success

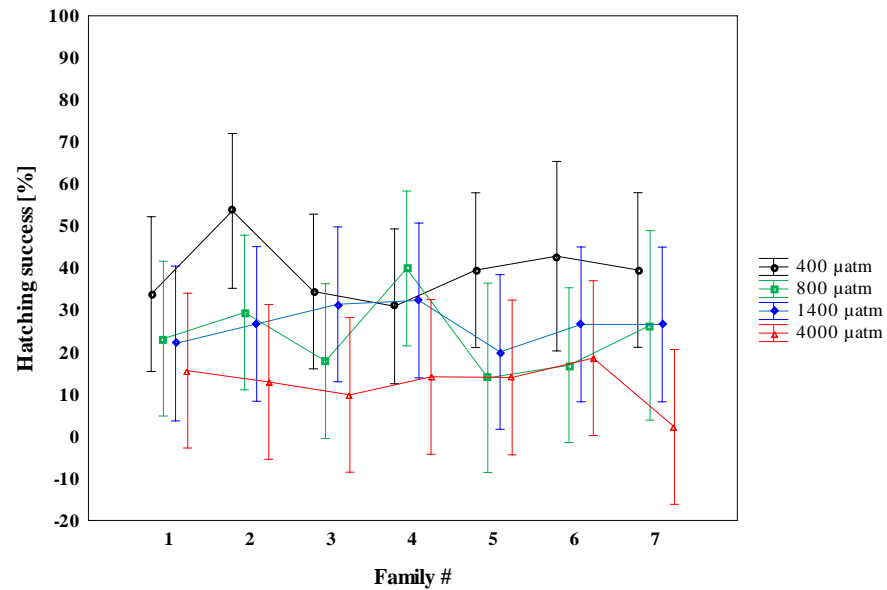


Figure 21: Plot shows no significant ($p = 0.86$, $F = 0.63$) interaction of $p\text{CO}_2$ and paternity on the mean percentages of hatched embryos. Error bars denote \pm confidence interval (CI) (95%) with $n = 3$ replicates.

Hatching success was defined as the percentage of hatched larvae which was calculated as the ratio of hatched larvae to the total number of viable embryos 19 h post fertilization (hpf). Hatched larvae were collected and counted every 24 hours. Hatching success showed neither a significant interaction between $p\text{CO}_2$ and paternity (Fig. 21), nor a significant difference between paternal responses when the $p\text{CO}_2$ effects were excluded (Fig. 22).

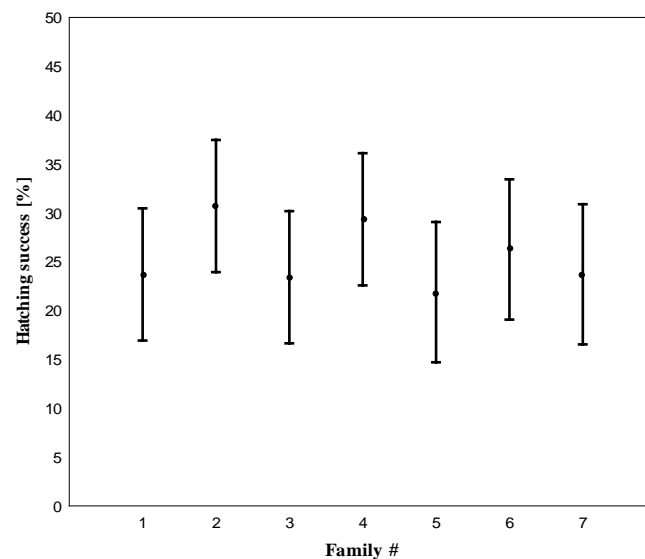


Figure 22: Mean hatching success related to paternal differences between 7 half sibling families without regard to $p\text{CO}_2$ concentration. No significant differences ($p = 0.63$, $F = 0.73$) among families were observed. Error bars denote \pm CI (95%) with $n = 3$ replicates.

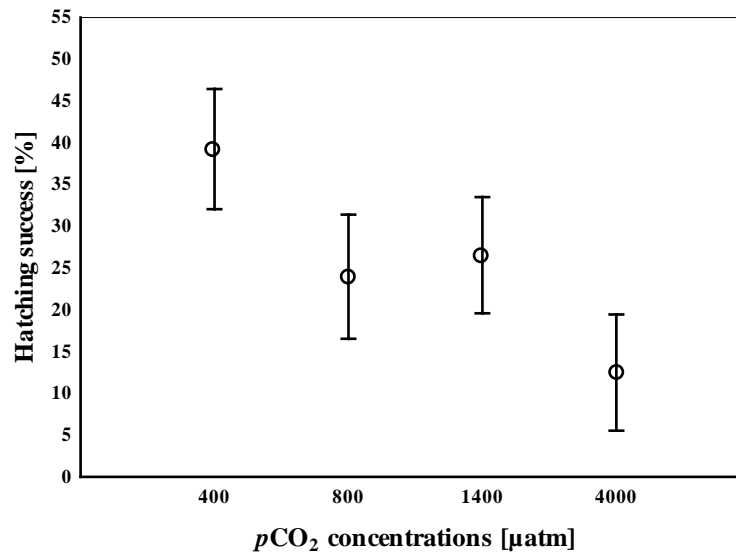


Figure 23: Mean hatching success related to $p\text{CO}_2$ concentrations at which all 7 families are combined per treatment. Showing significant differences ($p < 0.0001$, $F = 16.8$). Error bars indicate $\pm\text{CI}$ (95%) with $n = 3$ replicates.

Even if hatching success was not influenced by interaction or paternity, elevated $p\text{CO}_2$ concentrations significantly (multifactorial ANOVA, $p < 0.0001$, $F = 16.8$) affected the proportion of hatched larvae. Hatching success at 4000 μatm , was significantly ($p = 0.0002$) lower compared to the control, 800 μatm ($p = 0.03$), and 1400 μatm ($p = 0.02$). Hatching success ($\pm\text{SD}$) decreased with $p\text{CO}_2$ concentration and ranged from 39.0 ± 16.9 % in the control water to 12.5 ± 12.5 % in 4000 μatm (Fig. 23).

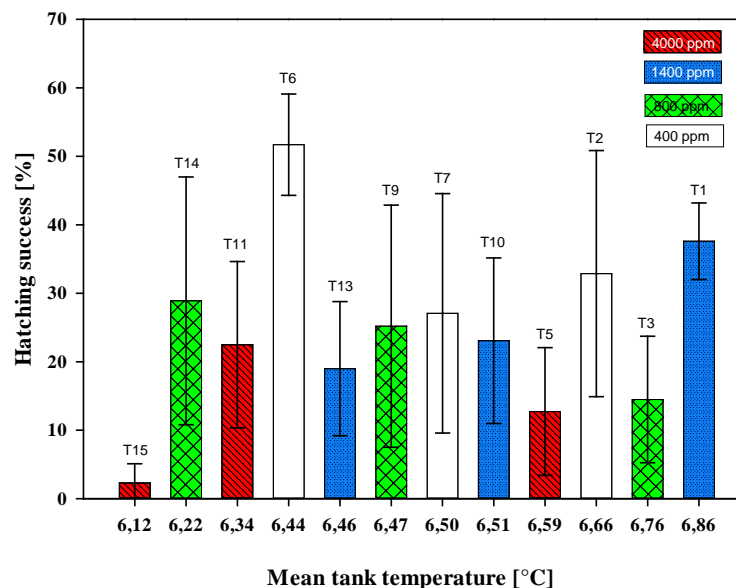


Figure 24: Hatching success in relation to differences in tank temperature recorded during the incubation time span. Each bar represents the mean percentage of 7 families in one tank. The tank number is noted on top of each bar. Error bars indicate \pm standard deviation with $n = 7$.

Differences in mean tank temperature ($\pm\text{SD}$) experienced during the incubation period, ranged from 6.12 ± 0.52 $^{\circ}\text{C}$ in tank 15 to 6.86 ± 0.72 $^{\circ}\text{C}$ in tank 1 and were not associated with hatching success (Fig. 24).

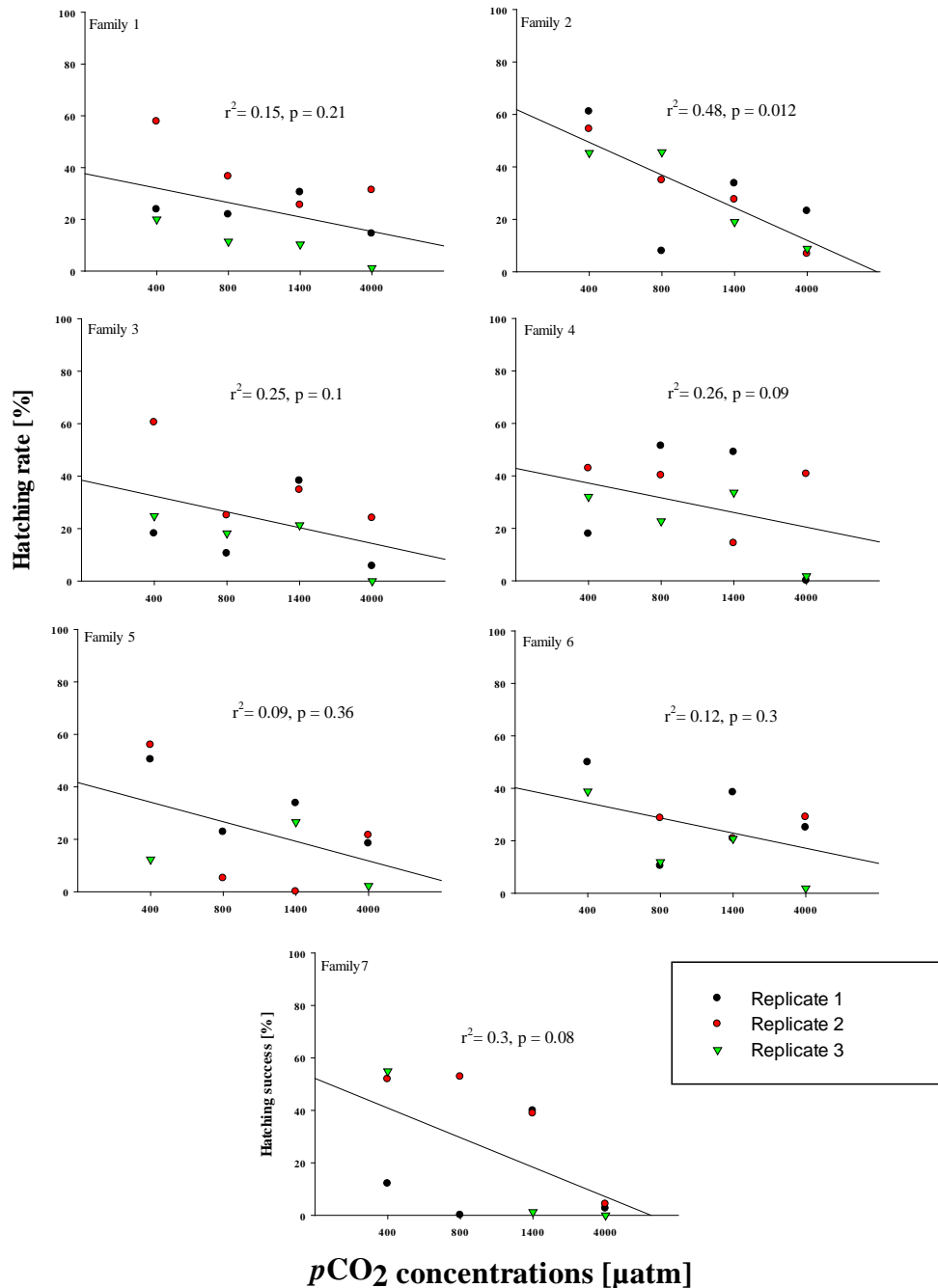


Figure 25: Hatching success per family as a function of $p\text{CO}_2$ concentration. Within each plot every data point represents a single replicate (Tank). The regression lines are just for visualization because treatments are given in categories but calculated values for (r^2) and (p) are given in each plot.

When looking at the families separately, the same pattern with decreasing hatching success with increasing $p\text{CO}_2$ concentration is found which underlines that hatching success is not or just very slightly influenced by paternity (Fig. 25).

3.5 Larval length

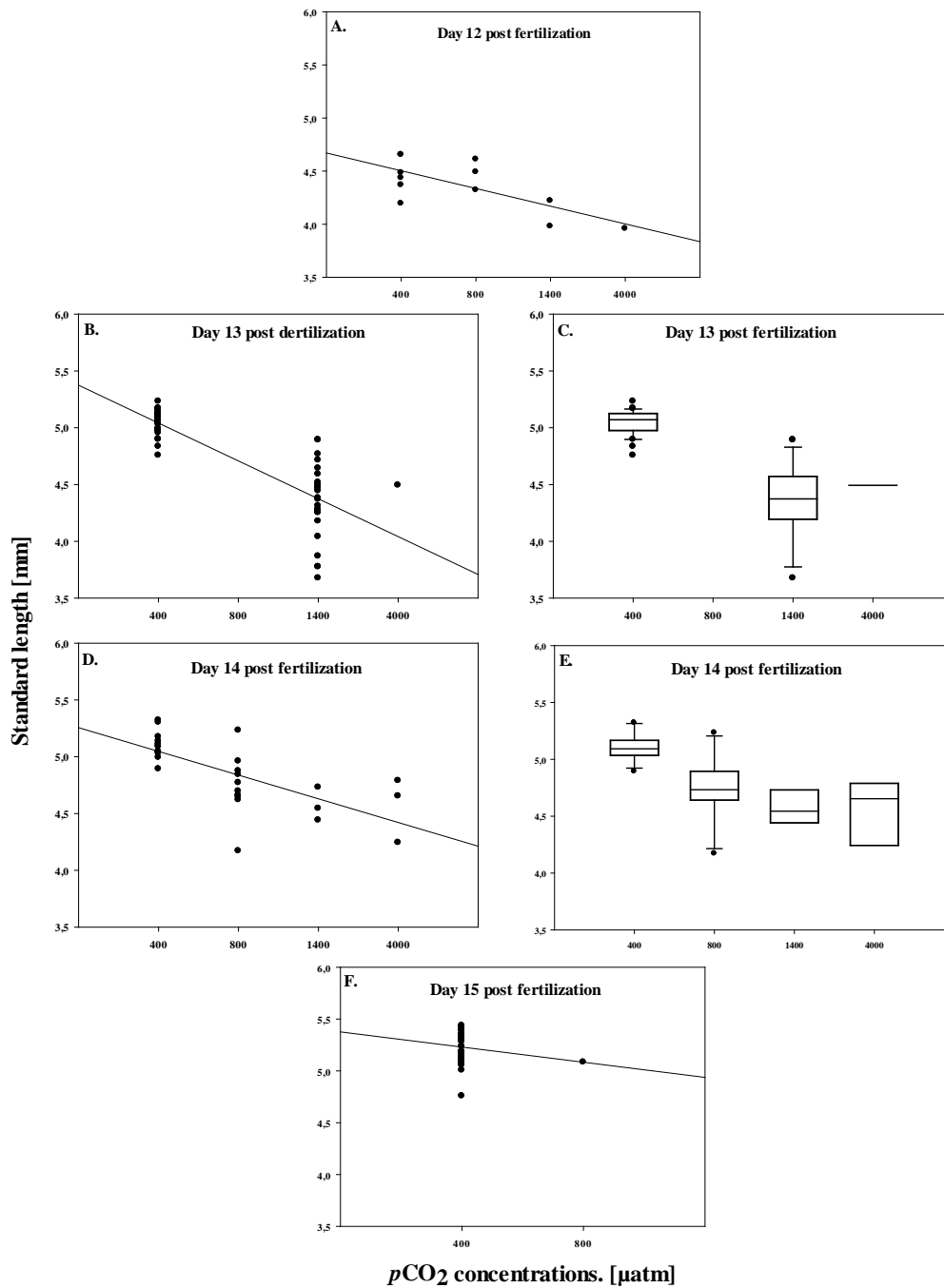


Figure 26: Larval standard length SL in relation to $p\text{CO}_2$ concentrations at different times post fertilization. Each point in (A), (B), (D), and (F) denotes a single larva with its SL [mm] while (C) and (E) shows the larval SL combined in box plots (box line denotes the median, the box boundary denote the 25th and the 75th percentile, Whiskers (error bars) indicate the 90th and the 10th percentiles). (B), (D), (C) and (E), respectively represent the days at which 50 % of larvae hatched. Regression lines: $r^2(\text{A}) = 0.51$, $r^2(\text{B}) = 0.67$, $r^2(\text{D}) = 0.49$, and $r^2(\text{F}) = 0.03$. Box plots are used for a better visualization of the data spread within the treatments.

No statistical analysis of the effect of treatment on standard length (SL) at hatch was conducted because of the low number of larvae especially in the high $p\text{CO}_2$ treatments, and the high variability among SL measurements.

Nonetheless, if not considering paternity and showing the single SL measurements per replicate, a decreasing SL pattern with increasing $p\text{CO}_2$ concentration becomes visible. The plots from day 13 and 14 post fertilization (these days were also defined as the days where more than 50 % of all larvae hatched) indicate the high variability between and within the control and the treatments very well with larvae that hatched at day 13 showing a more compact distribution within the control measurements, ranging from 4.75 – 5.23 mm ($\Delta_{\text{mm}} = 0.48$), than the wider length distribution in the 1400 μatm with SL values ranging from 3.68 – 4.89 mm ($\Delta_{\text{mm}} = 1.21$). The same pattern was observed on day 14 post fertilization between the control and 800 μatm treatments.

4 DISCUSSION

4.1 Experimental Setup

The circumstance that egg and embryo characteristics provided from the maternal side are more important for variability between the offspring than paternal contributions (Heath & Blouw, 1998; Burt *et al.*, 2010) could be a major reason to design experiments with female crossings when searching for responses to adverse environmental conditions. However, some recent studies highlighted the possible underestimation of paternal features when responses were just based on female crossings (Green & McCormick, 2005; Bang *et al.*, 2006; Probst *et al.*, 2006; Dahlke, 2011). Nonetheless, in cases of investigations on genetic adaptation potentials, like in our study, the high maternal variability among females could mask paternal contributions because of the strong influence of non-genetic influences on egg viability. Our crossing design with just one female used for producing seven half sib families eliminates such insecurities by keeping the maternal variation constant and guarantees that the differences in responses between families are transmitted by the paternal genetic input (excluding any experimental errors in how the experiment was carried out).

Maternal factors which could occur during gametes sampling and contribute to low egg quality include stress, extraordinary pressure on the abdominal tract during stripping, contamination of the eggs with urine or feces, incorrect storage of eggs after stripping, or a too long storage time between stripping and fertilization (Kjørsvik *et al.*, 1990). To exclude these potentials impacts and to collect a sufficient amount of viable eggs stripping procedures were carried out with high caution and respect. These included sedation of the female and the males before they were stripped and discarding the first and last eggs of the batch used. To minimize the effects of prolonged storage the fertilization was finished within one hour after stripping while the gametes were stored on ice and in a refrigerator ($\sim 5^{\circ}\text{C}$). Since eggs within 4 h and sperm until 8 h after stripping are still viable (dry and cold storage, $\sim 5^{\circ}\text{C}$) (Kjørsvik *et al.*, 1990; Trippel, 2003) storage related impacts in our experiment can be neglected.

Beside the quality of paternally forwarded genetic contribution environmental stress could affect non-genetic parameters like spermatogenesis and sperm motility of external fertilizing teleosts and therefore also has the ability to create impacts in fertilization success which was shown in several studies (Manning & Kime, 1985; Alavi & Cosson, 2005, 2006; Cosson *et al.*, 2008), but in this trial all males were kept under the same husbandry. Regarding sperm density which is also a very important factor for fertilization success (Rakitin *et al.*, 1999a; Trippel, 2003) irregularities were avoided by using closely the same amount of sperm for all crossings which was calculated based on the measured spermatocrit proportions (Rakitin *et al.*, 1999b). We used these proportions to fertilize the

eggs with approximately equal sperm ratios and used the dry fertilization method to gain the highest fertilization success (Trippel *et al.*, 2005). Negative impacts on embryo survival due to contamination with harmful particles like e.g. viruses or debris of dead embryos which are a breeding ground for bacteria were avoided by using clean water, and a relative to the water volume low density of embryos. In our setup, adverse impacts due to this threat could be neglected because of the fact that we cultured a maximum of 1400 eggs in 50 l of 0.35 μm filtered and UV treated seawater which was supplementary exchanged every second day or less. Our densities of eggs per volume of water were far below recommendations which are 150 eggs per 300 ml (Nissling, 2004) even after the change of incubation containers close to hatch from the tanks into the 600 ml beakers the egg density (maximum 100 eggs per beaker) never reached a harmful level. The deposit of particles was exacerbated by a steadily weak current in the tanks maintained by little pumps. Additionally, daily inspection of each petri dish under the microscope, regular exchange or cleaning of the dishes and net covers, and a relatively permeable net cover with a mesh size of 1 mm helped in maintaining good rearing conditions.

Non-natural light cycles could also affect the embryos after the eye differentiation (Kjørsvik *et al.*, 1990), thus to experience natural light conditions (~8-10 light) in the climate room we exchanged the opaque tarp which covered the tanks at the beginning of the incubation period by a clear transparent tarp when eye differentiation was recognizable. Salinity could be another harmful parameter affecting eggs and embryos (Kjørsvik *et al.*, 1990) but unfavorable salinity changes between or within the tanks were not observed during incubation (Table 7) thus differences in responses due to salinity could be excluded. Oxygen saturation, when measured, was always above 100 % but the circumstance of regular air/CO₂ injections and regular water exchanges, and the relative low stocking of embryos per volume of water underlines the suggestion that oxygen had no effect on mortality and therefore also none on hatching success.

Embryo development and survival is highly temperature dependent (Kjesbu, 1989) but regarding to the marginal mean tank temperature differences ($6.48 \pm 0.38^\circ\text{C}$) during in incubation, no significant correlation of temperature to time of hatching and hatching success was detected. Additionally, the possibility that brief temperature changes interact with effects induced by $p\text{CO}_2$ could be neglected because of the random tank distribution in the climate room.

4.1.1 $p\text{CO}_2$ conditions

The treatment $p\text{CO}_2$ concentrations in the seawater were achieved by aeration with a mix of air and CO_2 . These concentrations were controlled by constant pH measurements and regulated by further short time (3 sec) gas injections when needed. This kind of aeration with an air/ CO_2 mixture was used in many experiments and even recommended in the Guide to best practices for ocean acidification research and data reporting (Riebesell *et al.*, 2010). Henry's law describes the steady-state of partial pressures between the gas in air and the dissolved gas. This always ongoing equilibration affects mainly the high treatment tanks and by using a more or less open system a steady outgassing of CO_2 appeared which can cause slight differences between the predicted and the measured $p\text{CO}_2$, beside the analyzing errors (e.g. calibration of the pH sensors with non-seawater standards). Prolonged irregularities in $p\text{CO}_2$ could be excluded due to the continuous report of measured pH and daily calibrations. Absorption of exhaled CO_2 into the tanks could not be excluded especially the control water tanks which showed a slight pH decrease (from ~ 8 down to ~ 7.9 after a daily embryo sampling period) because of the small room size and the fact that sometimes the room was well-staffed (max. 3 persons). Nonetheless, a regular water change and the fact that the variabilities were relatively small in comparison with the natural $p\text{CO}_2$ fluctuations experienced in the ocean (Fig. 27) could weaken their impacts on egg, embryo and larval responses.

The use of an appropriate control water $p\text{CO}_2$ level near the naturally experienced scenario is an important issue in ocean acidification experiments dealing with physiological responses and is very often overlooked (McElhany & Shallin Busch, 2012). Regarding the environmental $p\text{CO}_2$ concentrations at Browns Bank, determined during a survey in the mid of April 2009 (Fig. 27 (A)) values between $250 \mu\text{atm}$ at the surface and up to $\sim 400 \mu\text{atm}$ at depth of 60 to 90 m where cod at these spawning regions release their eggs (Brander, 2005). This fits very well with our control water concentrations of about $\sim 400 \mu\text{atm}$. Since cod from these regions spawn from February to March (Brander, 2005) and ambient $p\text{CO}_2$ concentrations stay constant till March (Fig. 27 (B)), the experienced levels differ just very slightly from the $p\text{CO}_2$ concentrations measured in our control water and could lead to the assumption that Browns Bank spawners and their progeny have been reared at control conditions in the laboratory which reflect the conditions experienced in the wild at present day CO_2 concentrations.

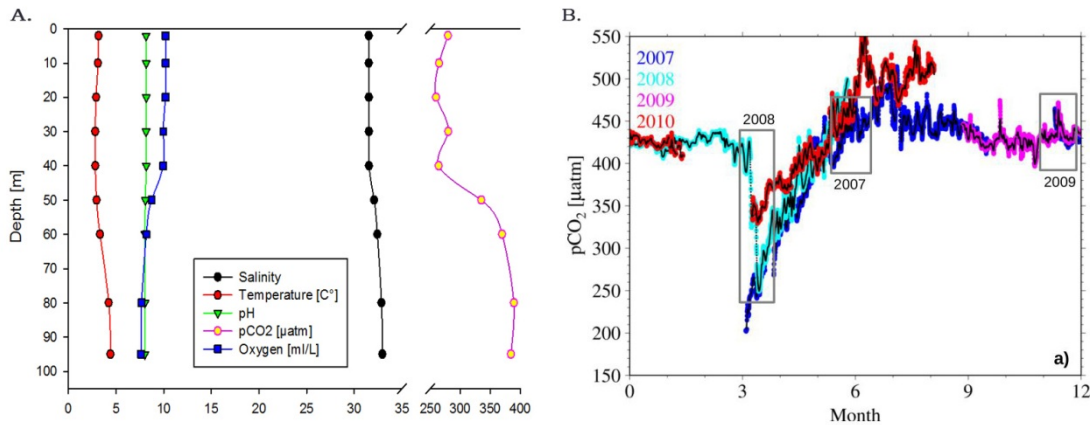


Figure 27: Hydrographic parameters from Browns Bank (65°28'6521"W; 42°26'9060"N) collected in mid April 2009. Data provided by Fisheries and Oceans Canada (2012) Atlantic Zone Monitoring Program and C_T and A_T values provided by Helmuth Thomas, Dalhousie University. B: Seasonal composite of available surface pCO₂ for the years 2007-2010 from the Scotian Shelf, identifying this region as a strong source for atmospheric CO₂ with a short period of CO₂ uptake during the spring bloom occurring from late March to early April. Grey boxes do not have any significance for this thesis. Taken from Thomas et al., (2012).

4.1.2 Sampling procedure

Embryogenesis in cod undergoes different stages of sensitivity with the highest mortality occurring shortly after fertilization until gastrulation (first 3 - 4 days) and then again 1 - 2 days before hatch (Bunn *et al.*, 2000). Because sampling procedures physically induced impacts beside the pCO₂ treatment they were minimized during these highly sensitive embryo stages by not transferring the embryos into new petri dishes, not changing the water which may be associated with slight temperature or salinity changes and storing the petri dish racks after removal of one petri dish always back into the tank water. The only time we treated the embryos and eggs within such a sensitive period physically was 19 h post fertilization by using a syringe to transport a sub-sample from the beakers to the petri dishes and while recording the images a slight increase in water temperature could have affected embryos and eggs. However, this happened at all treatment levels in all replicates.

Susceptibility regarding hypercapnic conditions in teleost fish is also dependent on developmental stages (Kikkawa *et al.*, 2003; Ishimatsu *et al.*, 2004; Melzner *et al.*, 2009a; Frommel, 2012) including the gametes and the subsequent early embryo development until the 1k cell stage. Especially these stages were in the focus of this study by activating the gametes already with hypercapnic water and starting to sample 19 h post fertilization.

The circumstance that not all petri dishes (families) provided hatching embryos because of a high mortality and a high variability between the family and treatment replicates made a statistical analysis for time to 50 % and larval length at hatch problematic. A higher density of eggs per petri dish could have been a solution for this issue but the reason not to fill more eggs into a dish was related to the imaging process 19 h post fertilization. A

higher density per petri dish would have resulted in several layers of eggs and made a later photographic analysis impossible but to obtain the number of viable fertilized eggs was crucial for further estimations of hatching success.

To exclude effects regarding a pattern in sampling, the investigated petri dishes during daily collections and enumerations were randomly chosen out of the randomly distributed tanks.

4.2 Effects on cod embryos

4.2.1 Gamete and early embryo estimations

Even small increases in seawater CO₂ concentration are mirrored via rapid diffusion in the bodies of water-breathing animals. Unicellular organisms like gametes in oviparous spawners experience relatively high changes in ambient $p\text{CO}_2$ of nearly 100 % because they are lacking an additional extracellular medium (Melzner *et al.*, 2009a) which could weaken the degree of CO₂ absorption. Since an epithelium located acid-base regulating mechanism is non-existent in fish eggs we could assume that responses to high $p\text{CO}_2$ are mainly depending on their intracellular buffer capacities which are traits forwarded by their parents. This could be a reason why CO₂ impacts on gametes differ between and within species such as a study on Baltic cod sperm showed no effects on motility in hypercapnic conditions (Frommel *et al.*, 2010) whereas white sturgeon (*Acipenser transmontanus*) and flatfish sperm motility were decreased (Ingermann *et al.*, 2002; Inaba *et al.*, 2003). Regarding Baltic cod a subsequent study on early life stages underlined their robustness to hypercapnic conditions (4200 μatm) with the conclusion that this could be attributed to adaptation (Frommel *et al.*, 2012b). Nonetheless, our results on Canadian cod the proportion of unfertilized eggs available 19 h post fertilization (Fig. 16 (A)) showed a strong pattern of a decreasing fertilization success with increasing $p\text{CO}_2$ and variability among families. But fertilization is, beside sperm quality (Rakitin *et al.*, 1999a) highly dependent on the egg quality which could be affected by stress, overripening, disturbances in ovulation procedure and mainly by maternal forwarded genetic or non-genetic egg features (Kjesbu, 1989; Kjesbu *et al.*, 1990; Kjørsvik *et al.*, 1990; Kjørsvik, 1994; Charmantier & Garant, 2005). Since we could exclude handling impacts and used just one female for the crossings which spawned eggs with a quality that led to high fertilization success especially in the control tanks (Fig. 15 (A)) egg quality as factor for the observed impacts on fertilization in the high treatments can be excluded. Regarding the variability in responses between families to hypercapnic conditions in the fertilization success and in the significant interaction responses in viable embryo estimations, we assume that the paternal genetic contribution could play a crucial role in Atlantic cod's vulnerability to hypercapnia associated with a potential for adaptive potential. This adaptive potential can be explained by the fact that the

father brings one half, beside the mother's part, of the cell nucleus containing genetic information which may be linked to the number and timing of appearance of chloride cells as these aid in acid base regulation. It remains unclear if this is under maternal control, paternal or both and how variable these are for evolution to act upon. Thus if male A passes a manual for an upregulated acid-base mechanism (the genetic coded instruction to the differentiating offspring cells to keep the focus on e.g. the production of ion-exchange proteins) and male B passes a manual to his offspring with the instruction to keep the focus on growth and not on acid-base regulation then offspring of male A could have advantages in hypercapnic condition while during normocapnic conditions offspring of male B has the edge over A. These differences could lead in worst case to lethal effects but also to a better adaptation which then also could make them more attractive for mate choice.

If we exclude the effects between families and calculate the mean from all families and out of the replicates a 24 % decrease of viable embryos 19 h post fertilization between 800 μatm and the control becomes visible (Fig. 13). Regarding the variability in hypercapnia sensitivity stages during early teleost development the embryos first cleavage stages seem to be the most vulnerable ones. This was proven in a study on four teleost fishes dealing with extreme high and lethal $p\text{CO}_2$ concentrations. Their responses were reported on the median lethal $p\text{CO}_2$ concentration (LC_{50} = 50 % of test animals die within 24 h). Such as e.g. the red sea bream (*Pagrus major*) showed an LC_{50} when exposed for 15 min of about 22.000 μatm during early embryo cleavage stages and when exposed for 24 h a LC_{50} of 13.100 μatm revealed. Comparing these values with later embryo stages it was reported that these were more robust and could be treated for 15 min at ~99.000 μatm to produce a 50 % death proportion after 24 h (Kikkawa *et al.*, 2003). After 19 h the embryos were close to the end of the cleavage period or short before the blastula period (starts after the 128 cell stage), respectively but still far away from the blastula stage (22 - 56 hours post fertilization (hpf), ~500 cells) where the transcription of their own mRNA starts (Hall *et al.*, 2004), which could be the first chance to react actively on biochemical gradients. This is underlined by many studies which reported a strong increase in transcription, expression and activity of the Na^+/K^+ -ATPase during early embryo development as it was shown in marine sea urchin (*Strongylocentrotus purpuratus*) where the major increase in Na^+/K^+ -ATPase activity occurs from blastula to gastrula stage (Leong & Manahan, 1997, 1999; Marsh *et al.*, 2000). Na^+/K^+ -ATPase is an active transmembrane protein which is responsible for an excessive energy demand during early embryo ontogeny while regulating the acid-base and ion concentrations (Melzner *et al.*, 2009a). This lack in active acid-base regulation during the investigated 19 hpf embryo stages could explain the relative high impacts we observed. However, this could not explain why the 800 μatm treatment revealed higher impacts compared to the 1400 and 4000 μatm $p\text{CO}_2$ concentrations which were still significantly different from the control but far above what an embryo could experience under actual and near future ocean acidification scenarios, and stays unanswered at the moment.

A direct significant single paternal effect on the proportion of viable embryos 19 h post incubation was not shown (Fig. 14) but the circumstance that these calculations were based on all treatments showing high variability could mask impacts on viable egg proportions based on the paternal differences. Paternal effects are indicative when comparing the proportions between the families within the treatments (Fig. 17 (A-D)) and support observations that paternity could affect teleost fertilization success as shown e.g. for Atlantic cod (Trippel & Morgan, 1994) or for European sea bass (*Dicentrarchus labrax*) (Saillant *et al.*, 2001).

4.2.2 Embryonic development and hatching success

Hatch initiation is not determined by a certain larval length, so its more an age or conditional controlled process (Geffen, 2002). Peak hatch (time to 50% hatch) between the treatments showed a difference of about less than a day. If we view these results from an ecological perspective; it is highly improbable that the observed results between the control and the treatments, could create any prey competition between different batches (match-mismatch scenario (Anderson, 1988)). Nonetheless, the circumstance that the mean values of hypercapnic treatments led to an earlier hatch fits well when we define hatching as a conditional induced event beside the knowledge that acid-base regulation (e.g. Na^+/K^+ -ATPase activity) correlates with the metabolic rate in teleost fishes (Melzner *et al.*, 2009a). Therefore, the more energy resources of the embryo yolk are used for acid-base regulation the less remains for tissue differentiation and growth. Additionally the yolk is faster depleted due to high metabolic rates and could lead beside the initiation to an earlier hatch to earlier starving when prey is not directly available. Same pattern was reported when higher metabolic rates were produced by elevated water temperatures correlating with faster development rates and leading to an earlier hatch (Geffen *et al.*, 2006).

After $\sim 13 \pm 1$ days in hypercapnic conditions hatching success was massively impacted by CO_2 with more than a 3-fold decrease between the 4000 μatm (12.5 %) and the control (39 %) treatment. Even the hatching proportions for the 800 and 1400 μatm were close to half of the control. These negative susceptibility changes to $p\text{CO}_2$ stress could be explained by the chronic exposure to high CO_2 concentrations and by possible lethal tissue damages in crucial internal organs. Kikkawa *et al.* (2003) reported a twofold increase within late embryo susceptibility when the exposure was prolonged from 15 to 360 min, they reported in LC_{50} which are “lethal” concentration responsible for 50 % mortality after 24 h and ranged from 15 min exposure with 98.000 μatm to 360 min at 48.800 μatm in Japanese whiting (*Sillago japonica*). If we assume that this linear increase in vulnerability maintains and we extend it to the incubation time span from our experiment of ~ 312 h we could evaluate our high mortality in the elevated $p\text{CO}_2$ concentrations until hatch and still have room left for evaluations even if the increase in susceptibility is sigmoidal. This pattern was shown on four marine teleost

species with differences in LC_{50} concentrations but all showed the same relation between increasing vulnerability and exposure time in embryo stages. Variability in such lethal responses between species are suggested and also already shown e.g. Baltic cod's hatching success was not affected by nearly the same pCO_2 concentrations we used (Frommel *et al.*, 2012b). However, Baltic cod early life stages and adults already experience relatively high and variable pCO_2 concentrations in their natural environment whereas Atlantic cod from the south Canadian Atlantic west coast inhabit a relatively stable CO_2 environment (Fig. 27).

The discussion on the causes for the massive impacts in hatching success are more speculative than based on knowledge about how chronic hypercapnic conditions affects crucial life functions in marine teleost embryos which are not available or just deficient. Nonetheless, a study on Atlantic cod larvae (max 45 days old) revealed lethal tissue damage in many internal organs when exposed to close to same hypercapnic conditions we used (Frommel *et al.*, 2012a). If these lethal organ damages are also responsible for the higher mortality in embryo stages we observed remains unclear but should be an aim for further studies.

Natural variation in larval length results from between-female differences in egg size and other genetic factors, and within-maternal factors, due to seasonal and annual environmental changes (Chambers & Leggett, 1996; Kjesbu *et al.*, 1996). Under stress-free conditions, if comparing body size of early hatchers with the size of the last hatched larva from the same batch, it seems that the size is not correlated to early or late hatching events, because earlier hatched smaller larvae grow faster after hatch and therefore could compensate for the size differences at hatch (Geffen, 2002). But if this balance could be maintained under biotic or abiotic stress is not obvious. The results of the present study showed a negative correlation of larval standard length (SL) with increasing hypercapnic stress which fits well into the early hatch observations and underlines the conclusion that a re-allocation in embryo tissue differentiation could have occurred. A decrease in SL in correlation to increased pCO_2 concentrations was also shown for Atlantic herring (Bodenstein, 2012) and in Japanese killifish (*Oryzias latipes*) (Tseng *et al.*, *in prep.*). From an ecological point of view – is the fact that larger larvae start earlier with the external food uptake, and that they contain more reserve substances to burn during longer periods of no or less food, could lead to a higher survival rate (Miller *et al.*, 1988; Kjorsvik *et al.*, 1990; Kjesbu *et al.*, 1996). Additionally, larger larvae are better swimmers and therefore have increased chances to escape from predators accompanied by advanced food searching and hunting abilities (Miller *et al.*, 1988). Projecting these facts to our results in which the larvae hatched under elevated pCO_2 conditions were smaller in body size, and if we add the earlier hatch of these larvae assuming this happens because of a depleted yolk used up when trying to actively compensate the high extra- and intracellular pCO_2 concentrations, we could suppose impacts in larval survival in future ocean acidification. Nonetheless, projecting the results of this study to wild populations and their possible impacts governed by future climate change should always be done with caution because climate change stress occurs at

much lower speed than we exposed the organisms to. Potential acclimatization could weaken impacts induced by environmental changes.

We observed highly vulnerable stages from gametes to early cleavage stages showing interactions with paternity and high impacts in embryo survival after 19 h. This can be explained by the hypothesis of lacking extracellular buffer capacities in gametes/embryos and a lack in active acid-base regulation in early cleavage stages. After 13 days incubation embryo survival is significantly decreased and correlates with increasing treatment concentrations. This is despite the assumption of a decreasing vulnerability due to an increasing differentiation of active acid-base mechanisms like epidermal chloride cells. Additionally no family interaction in hatching success could be observed. A possible explanation could be the chronic exposure to high $p\text{CO}_2$ concentrations which could overwhelm the energy demanding chloride cells. Impacts in hatching success were accompanied by earlier hatch and smaller larval body length which could be explained by higher metabolic rates produced by active acid-base regulation resulting in faster yolk depletion and therefore to an earlier hatch accompanied with less time and energy for growth. On the other hand, another study Atlantic cod larvae had a positive correlation of growth to elevated carbon dioxide concentrations with the assumption that body volume is correlated to an increase in cutaneous acid base regulation (Frommel *et al.*, 2012a) which also governs the metabolic rates. However this increase in surface-to-volume ratio is also accompanied by increase in CO_2 diffusion area and thereby may not be a proper response to hypercapnia and could be the reason for the observed organ impacts (Frommel *et al.*, 2012a). In comparison to the prior hatch stage responses which correlated negatively with increasing hypercapnic conditions, post hatch larvae have the ability for endogenous food consumption which helps to deal with the high metabolic rates. But in embryos with a finite amount of energy CO_2 stress could lead to a re-allocation in embryo differentiation such as a reduced growth even during higher metabolic rates because energy is used to export ions from the tissues and/or to increase the density of cutaneous acid-base regulation mechanisms (Cameron & Iwama, 1987).

4.2.5 Synergistic stressors

Most studies investigating the potential impacts of adverse environmental conditions on marine organisms mostly focus on just one parameter but regarding to environmental stress experienced under natural conditions these parameters do not occur alone. To create more realistic models e.g. for ocean acidification (OA) some stress supporters should also be included in future studies. Regarding to interaction responses of OA, hypoxia, salinity, and temperature on the marine biosphere some few publications are present which often just discuss the impacts of two parameters at the same time (Pörtner, 2006, 2008; Munday *et al.*, 2010; Landes & Zimmer, 2012; McCulloch *et al.*, 2012; Melzner *et al.*, 2012b; Sunda & Cai, 2012). Same

experimental designs could be observed in studies dealing with marine fish and responses related to environmental conditions whereas cumulative impacts including OA are very rarely discussed (Munday *et al.*, 2009; Pörtner & Peck, 2010; Nowicki *et al.*, 2012) such an example could be the interaction of hypoxia with OA in oceanic waters but actual it is neglected. However, with increasing ocean acidification hypoxic zones in the oceans coastal regions become more important (Melzner *et al.*, 2012b). Water temperature seems to create the strongest effects on fish physiology like impacts on gametogenesis, gonadal growth, fecundity and egg viability, larval size, larval growth (Kjørsvik *et al.*, 1990; Burt *et al.*, 2010; Dahlke, 2011; Hüsey *et al.*, 2012; Voss *et al.*, 2012). This vulnerability to elevated temperature in combination to predicted oceanic temperature rises of $\sim 2^{\circ}\text{C}$ to 3.5°C induced by climate change could force marine organisms like Atlantic cod to leave actual spawning grounds northbound to colder habitats (Drinkwater, 2005; Fogarty *et al.*, 2007). Some of these northbound regions are already exposed to high $p\text{CO}_2$ concentration caused by the ability of cold water to absorb higher amounts of carbon dioxide and high primary production (Fabry *et al.*, 2009). A possible response of a cold water adapted fish like Atlantic cod to hypercapnic conditions could be an elevated sensitivity to warmer temperatures. But on the other hand it could have a positive effect on fish stocks because of the circumstance that the arcto-boreal regions with temperature close to the low temperature tolerance limit the predicted thermal rise and could create comfortable water temperatures (Drinkwater, 2005). Fish nutrition is also expected to be impacted by climate change such as shifts in plankton or even die-offs such as reported impacts in Pacific oyster larva densities at the west coast of the US (Barton *et al.*, 2012) which could affect fish conditions and therefore be responsible for shifts in fish habitats or in worst case to a decrease in their biomass (Beaugrand *et al.*, 2008; Fabry *et al.*, 2008; Guinotte & Fabry, 2008; Kirby *et al.*, 2009; Pörtner & Peck, 2010). Additionally climate change leads to more extreme weather conditions like storms which could increase the vertical mixing of the surface layers resulting in thinning out the nutrition particles responsible for a vital feeding (Lasker, 1975) or could transport pelagic eggs and larvae into unfavorable regions (Anderson, 1988). All these parameters have the potential to arise from future climate change and could narrow physiological tolerance limits to the other parameters and create even stronger impacts on marine fish stocks whereas commercial important fish stock are already in a crucial level and therefore highly vulnerable.

4.2.6 Acclimatization and adaptation to high $p\text{CO}_2$

Early ontogeny stages are very sensitive and vulnerable to environmental stress and dependent on the parental induced configuration. This dependence provides scope for a wide variety of resilience and adaptive responses controlled by the parents (Kamler, 2005). But important questions are how possible acclimatization could affect chronic physiological impacts caused by environmental stressors and how rapid adaptation could

happen?! The problem with such kind of studies is that they just persist for weeks to months but climate change is rising very slowly. Especially species with long generation turnover cycles don't have the chance to genetically adapt in time spans over several hundreds of years (Melzner *et al.*, 2009a). However, to estimate these impacts and sensitivities, on the species level, it will help to extend the understanding and looking at ecological responses to e.g. ocean acidification. Additionally this will provide useful information for aquaculture and fisheries management. The basic requirement for adaptation is variance e.g. within species responses to environmental impacts. A broad variety of responses to hypercapnic conditions within and between species levels ranging from highly vulnerable like reported in this study to very robust species like Baltic cod (Frommel *et al.*, 2012b) has been reported. One of the most extreme examples might be the cyprinid teleost fish Osorezan dace (*Tribolodon hakonensis*) which inhabits a highly acidic (pH of ~3.5) Japanese lake called Usoriyama. The dace is well adapted to this high acidified water by containing high density of gill tissue located chloride cells which contain high concentrations of ion exchange proteins (Kaneko *et al.*, 1999; Hirata *et al.*, 2003). So far very few studies placed their focus on potential acclimatization when exposed to elevated $p\text{CO}_2$ such as Melzner *et al.*, (2009b) who investigated the swimming performance after adult Atlantic cod was reared for 4 to 12 month in $p\text{CO}_2$ concentrations of 3000 and 6000 μatm . They showed no significant signs in swimming burst but elevated ion-exchange protein expression in the high treatments. Another possible adaptation could be a rise in buffer production which could be essential especially in early embryo cleavage stages. On the other hand, adaption or acclimation could increase sensitivities to parameters to which organisms were not exposed to. Thus, this could also have advanced the results of this study when assuming that captive fish clones cultured over years or decades under stable conditions and mainly concentrating on offspring production may produce different levels of responses compared to the wild stocks.

5 CONCLUSIONS

The results suggest sensitivities in early stages of Atlantic cod embryos from the Canadian shelf to hypercapnic seawater and a paternal genetic adaptation potential to future ocean acidification scenarios. If transferred to wild populations the suggested adaption potential could be forced by maternal genetic adaption, migration and other mechanisms. Such an adaptation could already be responsible for reported differences between early life stages of Baltic cod (Frommel *et al.*, 2012b) and Atlantic cod sensitivities to ocean acidification. Additionally, acclimation could decrease climate change driven responses like it was reported for hypercapnic conditions in Atlantic cod after 12 month incubation (Melzner *et al.*, 2009b) or even an acclimation after two generations to thermal stress like shown for an tropical reef fish (Donelson *et al.*, 2011). Thereby, the exposure of organisms to a rapid “shock” $p\text{CO}_2$ change like it was conducted in the present study, compared to a predicted slower rise of $p\text{CO}_2$ due to ocean acidification, could possibly lead to physiological reactions that are stronger than what would be expected if a wide range of potential acclimation mechanisms had time to develop. It is assumed, for example, that epigenetic driven phenotypic differences in response to stress could occur already after one generation (Anway *et al.*, 2005). However, the results of the present study should be observed under the circumstance that just one environmental parameter, $p\text{CO}_2$, was investigated, assuming that an addition of further relevant parameters simultaneously (e.g. warming, hypoxia or nutrition) would lead to more significant conclusions to climate change impacts on the species-level.

Our reported sensitivities are a good starting point for further intensified research on vulnerable early life stages of e.g. Atlantic cod. Future work should include prolonged acclimation periods, additional environmental parameters like warming and paternity in the focus of experimental designs.

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Eidesstattliche Erklärung

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